Developing Molecular and Physiological Markers for Barley Breeding for Waterlogging Tolerance by Targeting Root Ionic Homeostasis

By

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Declarations

Declaration of Originality

The thesis contains no material which has been accepted for the degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Chapter 3

Abbreviations

ADH	Alcohol dehydrogenase
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSM	Basal salt medium
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
DArT	Diversity array technology
DH	Doubled haploid
DNA	Deoxyribonucleic acid
FDA	Fluorescein diacetate
Gd^{3+}	Gadolinium chloride
GORK	Guard cells outward rectifying K^+ channel
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
KIR	K^+ inward-rectifying channels
KOR	K^+ outward-rectifying channel
LIX	Liquid ion exchangers
MIFE	Microelectrode ion flux estimation
MP	Membrane potential
MQM	Multiple QTL model
NBT	Nitro-blue tetrazolium
NSCC	Non-selective cation channel
$O_2^{\cdot -}$	Superoxide radical

OH^-	Hydroxyl ion
PCD	Programmed cell death
PI	Propidium iodide
PM	Plasma membrane
PMHA	Plasma membrane H^+ ATPase
QTL	Quantitative trait locus
RILs	Recombinant inbred lines
ROL	Radial oxygen loss
ROS	Reactive oxygen species
SKOR	Stelar outward-rectifying K^+ channel
SOD	Superoxide dismutase
TEA	Tetraethylammonium chloride
TX9425	Taixing 9425
Vanadate	Sodium orthovanadate
V-ATPase	Vacuolar type H^+-ATPase

Abstract

Waterlogging is a serious environmental threat worldwide that severely limits agricultural production. Waterlogging stress adversely affects 10% of the global land area and annual financial losses to agricultural crop production are estimated to exceed €60 billion. Many regions of the world (e.g. Australia, China) are regularly affected by waterlogging stress. Barley ranks fifth amongst all crops in dry matter production in the world. In Australia, most of the barley cultivars are waterlogging sensitive. In most cases, plants are grown on duplex soil, which has a layer of sandy soil over a comparatively water-resistant base of clay soil. Therefore, the continued rainfall events can lead to increasing water tables in the root zone. Waterlogging is a complex trait that conferred by several physiological and biochemical mechanisms. While the major attention of plant breeders was on traits related to oxygen supply and retention (radial oxygen loss; aerenchyma formation; etc), traits related to plant's ability to maintain ionic homeostasis under waterlogged conditions received much less attention. Therefore, the main objectives of this study were (1) to investigate the effects of oxygen deprivation on intracellular K^+ signalling and homeostasis, and its potential roles in acclimation to oxygen-deprived conditions in barley; (2) to develop reliable screening protocols for evaluation of some key physiological traits conferring waterlogging stress tolerance which can be used in breeding programs; (3) to link waterlogging tolerance with K^+ retention, membrane potential maintenance and ROS stress tolerance by using a QTL approach; (4) to link these traits with other abiotic stresses such as salinity in order to make the deep understanding of tolerance mechanisms in barley.

The major constraint that plants undergo in waterlogged conditions is the inadequate supply of oxygen to submerged parts. Oxygen depletion under waterlogged conditions results in a compromised operation of H^+ -ATPase, with strong implications for membrane potential maintenance, excessive ROS accumulation, cytosolic pH homeostasis, and transport of major nutrients across membranes. The above effects, however, are highly tissue-specific and time-dependent, and the causal link between hypoxia-induced changes to cell's ionome and plant adaptive responses to hypoxia is not well established. This work aimed to fill the above gap and investigate the effects of oxygen deprivation on K^+ signalling and homeostasis in plants and potential roles of GORK (depolarization-activated outward-rectifying potassium) channels in plant adaptation to oxygen-deprived conditions in barley. The significant K^+ loss was observed in roots exposed to hypoxic conditions; this loss correlated with the cell's viability.

The stress-induced K^+ loss was stronger in the root apex immediately after stress onset but became more pronounced in the root base as the stress progressed. The amount of K^+ in shoots of plants grown in waterlogged soil correlated strongly with K^+ flux under hypoxia measured in laboratory experiments. Hypoxia-induced membrane depolarization was less pronounced in the tolerant group of cultivars. The expression of *GORK* was down-regulated by 1.5-fold in mature root while upregulated by 10-fold in the apex after 48 h hypoxia stress. Taken together, our results suggest that GORK channel plays a central role in K^+ retention and signalling under hypoxia stress and measuring hypoxia-induced K^+ fluxes from the mature root zone may be used as a physiological marker to select waterlogging tolerant varieties in breeding programs.

Waterlogging and salinity are two major abiotic stresses that could occur simultaneously and hamper crop production world-wide resulting in multibillion losses. Plant abiotic stress tolerance is conferred by many interrelated mechanisms. Amongst these, the cell's ability to maintain membrane potential is considered to be amongst the most crucial traits, a positive relationship between the ability of plants to maintain highly negative membrane potential and its tolerance to both salinity and waterlogging stress. However, no attempts have been made to identify quantitative trait loci (QTL) conferring this trait. In this study, the microelectrode MIFE technique was used to measure the plasma membrane potential of epidermal root cells of 150 double haploid (DH) lines of barley (*Hordeum vulgare* L.) from a cross between a Chinese landrace TX9425 and Japanese malting cultivar Naso Nijo under hypoxic conditions. A major QTL for the membrane potential in the epidermal root cells in hypoxia-exposed plants was identified. This QTL was located on 2H, at a similar position to the QTL for waterlogging and salinity tolerance reported in previous studies. Further analysis confirmed that membrane potential showed a significant contribution to both waterlogging and salinity tolerance. The fact that the QTL for membrane potential was controlled by a single major QTL illustrates the power of the single-cell phenotyping approach and opens prospects for fine mapping this QTL.

A reduced concentration of oxygen in waterlogged soils leads to oxygen deficiency in plant tissues, resulting in an excessive accumulation of ROS in plants. This ROS accumulation under waterlogged conditions also contributes to limit agricultural production in low-lying rainfed areas worldwide. To identify QTL for ROS tolerance in barley, 187 double haploid (DH) lines from a cross between TX9425 and Naso Nijo were screened for superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) accumulated under hypoxia stress. In our experiment, we

showed that quantifying ROS contents after 48 h hypoxia could be a fast and reliable approach for the selection of waterlogging tolerant barley genotypes. A major QTL on chromosome 2H was identified for both $O_2^{\cdot-}$ (*QSO.TxNn.2H*) and H_2O_2 (*QHP.TxNn.2H*) contents. This QTL was located at the same position as the QTL for the overall waterlogging and salt tolerance reported in previous studies, explaining 23% and 24% of the phenotypic variation, for $O_2^{\cdot-}$ and H_2O_2 contents, respectively. The analysis also showed a causal association between ROS production and both waterlogging and salt stress tolerance. The markers associated with this QTL could potentially be used in future breeding programs to improve waterlogging and salinity tolerance.

Taken together, the results of this work showed that hypoxic conditions caused a significant loss of K^+, in a time- and genotype-specific manner. This has affected cell viability and overall plant tolerance. The genotypic difference in waterlogging stress tolerance in barley was conferred by the differential ability to regulate voltage-gated K^+-permeable channels (GORK) in the mature root epidermis. A strong positive correlation between the ability of mature zone cells to retain K^+ and the overall waterlogging stress tolerance was found, making it possible to recommend using this method as a physiological marker for breeding plants for waterlogging stress tolerance. A major QTL for membrane potential maintenance under hypoxia stress was identified on chromosome 2H using cell-based phenotyping involving microelectrode MIFE technique. Another important finding of this work is the identification of two major QTL for both $O_2^{\cdot-}$ and H_2O_2 accumulation for hypoxia on Chromosome 2H. Interestingly, the QTL for membrane potential and ROS is located at a similar position to that for waterlogging and salinity tolerance on chromosome 2H. The fact that these QTL are detected at a similar position of chromosome 2H indicates a specific mechanism for different stress tolerances including waterlogging and salinity tolerance. Future work should be focusing to fine map these QTL and use this gene in pyramiding different tolerance mechanisms in breeding programs.

Key words: Barley, chromosome 2H, GORK**,** H^+-ATPase, H^+-PPase, *Hordeum vulgare* hypoxia, ionic homeostasis, membrane potential, potassium, QTL mapping, ROS, signaling, viability staining, waterlogging tolerance

Chapter 1

General introduction

1.1 Waterlogging as an issue

Waterlogging is a serious worldwide environmental problem that limits agricultural production in low-lying rainfed areas. This problem is predicted to rise due to the increased frequency of extreme flooding events during this century (Wollenweber et al., 2003; Seneviratne et al., 2012). Waterlogging stress adversely affects 10% of the global land area (Setter and Waters, 2003) and annual financial losses to agricultural crop production are estimated to exceed €60 billion (Dobrovičová et al., 2015). Various regions of the world (e.g. Australia, China) are regularly affected by waterlogging stress due to heavy rainfall, poor soil structure, inadequate drainage systems and subsoil compaction (Samad et al., 2001; Collaku and Harrison, 2002). Waterlogging stress inhibits the gas exchange rate between plant tissues and atmosphere because the diffusion rate of gases is 10000 times slower in water than in air (Kader and Saltveit, 2003). As a result, the level of oxygen in soil drops quickly from 230 nmol m^{-3} (well-drained soil) to 50 nmol m^{-3} (hypoxic) (Turner and Patrick, 1968) or may even result in a complete absence of oxygen (anoxia), due to increased microbial activities (Ponnamperuma, 1984). These oxygen-deprived conditions result in plant roots switching from aerobic respiration to an anaerobic fermentation process, reducing the rate of energy production (Gibbs and Greenway, 2003; Branco-Price et al., 2008).

Waterlogging stress, due to the lack of oxygen, imposes significant changes in the soil physiochemical profile; this includes soil pH and redox potential (ŠImek and Cooper, 2002; Pezeshki and DeLaune, 2012). Further reduction in the concentration of available oxygen affects the microbial activities in the soil (Chan et al., 2008), which eventually can lead to a reduced abundance of oxidized elements such as NO_3^-, SO_4^{2-} and Fe^{3+} and elevated levels of reduced compounds (e.g. Mn^{2+}, Fe^{2+}, H_2S, NH_4^+). An accumulation of the latter compounds is potentially toxic for plants during the long-term exposure to waterlogging stress (Ponnamperuma, 1984; Lucassen et al., 2000; Jackson and Colmer, 2005; Shabala, 2011). Meanwhile, reactive oxygen species (ROS) are produced and accumulated in excessive amounts during the onset of oxygen shortage and very often more upon reoxygenation (Blokhina et al., 2003; Jackson and Colmer, 2005). Several ROS may be produced under

flooding stress; among these, superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are very reactive, causing serious damage to lipids, proteins and nucleic acids (Blokhina et al., 2003; Yiu et al., 2009). In addition, hypoxia also limits the availability of required energy to fuel the H^+-ATPase pumps; the transport of nutrients from roots to shoots is also severely disturbed under waterlogged conditions (Smethurst et al., 2005; Colmer and Voesenek, 2009), which consequently limits the plant growth and yield (Bailey-Serres and Voesenek, 2008; Elzenga and van Veen, 2010). Waterlogging also badly impacts stomatal conductance and photosynthesis rate (Van Loc et al.; Malik et al., 2001) leading to a severe reduction in the growth and yield. Few plant species showed exceptional tolerance to waterlogging stress. Plant tolerance to waterlogging depends on multiple factors such as genotypic makeup of cultivars (Setter et al., 1999; Khabaz-Saberi et al., 2006), plant development stage at a time of waterlogging onset (Trought and Drew, 1980; Setter and Waters, 2003), and the duration of waterlogging stress (Kozlowski, 1997; Malik et al., 2002).

1.2 Waterlogging and barley production

Barley is considered more susceptible to waterlogging compared with other cereal crops. At the same time, barley possesses a significant variability in waterlogging tolerance between genotypes (Setter et al., 2008; Romina et al., 2014). Due to its sensitivity to waterlogging stress barley experienced considerable yield losses. It is estimated that barley production reduces by 20-25% in total, but the loss may exceed 50% due to waterlogging depending on the duration of stress and the stage of crop development at the time when stress occurs (Setter and Waters, 2003; Ahmed et al., 2012). Keeping in mind the potential costs of a drainage work required for fixing the problem of waterlogging (Smedema et al., 2004), developing waterlogging tolerant genotypes may be a sustainable solution to reduce yield losses to barley production.

To achieve waterlogging tolerance in barley, various physiological and agronomic mechanisms were targeted (Takeda and Fukuyama, 1986; Pang et al., 2006; Zhou et al., 2007; Xue et al., 2010; Mano and Takeda, 2012; Zhou et al., 2012). Several genetic linkage maps have been used in these studies including restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and diversity array technology (DArT). These maps have permitted the detection of quantitative trait loci (QTL) for several traits in barley. Furthermore, this makes it possible to use marker-assisted selection (MAS) as an efficient way to bring waterlogging tolerance into commercial barley varieties.

To date, several QTLs have been identified for waterlogging tolerance based on different physiological and agronomic traits. QTLs associated with waterlogging tolerance were mapped by targeting the germination rate (Angaji et al., 2010), total root dry weight (Naz et al., 2014), chlorophyll damage index (Bertholdsson et al., 2015), grain yield (Zaidi et al., 2015), leaf chlorosis (Li et al., 2008; Ma et al., 2015), survival rate (Ma et al., 2014), and photosynthetic characteristics (Pearson et al., 2011), using the whole-plant based phenotyping. In recent studies, more directly related traits to waterlogging tolerance have also been selected to identify QTLs such as root porosity (Broughton et al., 2015), adventitious roots development (Zhang et al., 2017) and aerenchyma formation (Mano et al., 2012; Zhang et al., 2015; Zhang et al., 2016). However, none of the above-given findings led to any major breakthrough in producing stress-tolerant barley genotypes.

Several reasons may explain this (Arzani and Ashraf, 2016). First, the statistical testing of null hypotheses (for example no QTL) is deeply embedded in the probability theory and conditions that create error variance leads to threats to statistical conclusion validity. The LOD threshold value for avoiding a false positive with a given confidence, say 95%, depends on the number of markers and the length of the genome. Consequently, the literature describing QTL analyses might contain false-positive QTLs at the too high rate. The second major reason is the genotype (QTL) by environment interaction which often confounds with main effects of a QTL. This is specifically true for all field-based studies. Next, quantitative genetic models are often based on certain (unrealistic) assumptions and have strong background dependency.

From a physiological point of view, the major shortfall is that in nearly all cases the above phenotyping has been conducted at the whole-plant level, so each of the measured traits was conferred by multiple (and often unrelated) contributing mechanisms. As a result, multiple QTLs have been reported for each of these traits. For example, fourteen QTLs were associated with leaf chlorosis on chromosomes 1H, 2H, 3H, 4H, 5H, 6H, 7H for waterlogging tolerance (Li et al., 2008; Xu et al., 2012; Zhou et al., 2012) and ten QTLs associated with plant height on chromosomes 1H, 2H, 4H, 5H, 7H for yield component (Li et al., 2005; Xue et al., 2010; Chutimanitsakun et al., 2011). The other reason is that very often the phenotypic indices used are not directly related to the mechanisms targeted and are, therefore, misleading. Hence, it seems that the real progress in plant breeding can be achieved only when plant phenotyping will directly target more closely related contributing mechanism at the cellular level. As shown

in the next chapter, plant ability to maintain intracellular potassium homeostasis is essential for plant signalling and adaptation to hypoxia stress, making the scope for this work.

1.3 Aims of the project

The aims of this research were four-fold:

1. To investigate the effects of oxygen deprivation on intracellular K^+ signalling and homeostasis and its potential roles in adaptation to oxygen-deprived conditions in barley.

2. To develop reliable screening protocols for evaluation of some key physiological traits conferring waterlogging stress tolerance, which can be used in breeding programs.

3. To link waterlogging tolerance with K^+ retention, membrane potential maintenance and ROS stress tolerance by using a QTL approach.

4. To link these traits with other abiotic stresses such as salinity in order to make a deep understanding of tolerance mechanisms in barley.

The above aims were addressed in a series of glasshouse and laboratory experiments conducted between 2014 and 2017.

1.4 Outline of the chapters

This thesis consists of six chapters.

Chapter 1. General introduction.

Chapter 2. Literature review

Chapter 3. The ability to regulate voltage-gated K^+-permeable channels in the mature root epidermis is essential for waterlogging tolerance in barley. This chapter describes how the $^+$ retention ability in roots is strongly and positively correlated with overall waterlogging tolerance in barley.

Chapter 4. Cell-based phenotyping reveals QTL for membrane potential maintenance associated with hypoxia and salinity stress tolerance in barley. A major QTL for membrane

potential maintenance associated with hypoxia and salinity stress tolerance in barley was identified.

Chapter 5. Identification of QTL related to ROS formation under hypoxia and their association with waterlogging and salt tolerance in barley. Two major QTLs for ROS production were identified and linked with waterlogging tolerance in barley.

Chapter 6. General discussion and future prospects.

Chapter 2

Literature Review

2.1 Major constraints imposed by waterlogging

2.1.1 Oxygen Deprivation

Deprivation of oxygen (O_2) is the main constraints imposed by the waterlogging stress in plant roots. Over the years, numerous breeding programs consistently targeted the plant's ability to retain higher O_2 level or increase the ability of plants to an adequate supply of O_2 to roots. Under the longer periods of waterlogging stress, chemical and biological processes continue until the little amount of O_2 is available (Gambrell et al., 1991). An important feature of flooding proceedings is the change of levels of three gases, O_2, CO_2 and ethylene. It happens due to 10000 times lower diffusion rate of gasses with the rate being about 10^4 in water as compared to 10^5 in the air. (Fukao and Bailey-Serres, 2004; Bailey-Serres and Voesenek, 2010). Waterlogging has very little or no impact on the growth of rice, as semi-aquatic species, as rice is capable of forming gas channels in plant parts in the air and parts submerged in water as well (Bailey-Serres et al., 2012; Jackson and Drew, 2012). After the few hours or days of onset of waterlogging stress, oxygen levels can be depleted depending on the energy availability and microbial activity (Gambrell et al., 1991; Colmer, 2003). The concentration of oxygen rapidly drops in soil solution from 230 m^{-3} in the well-aerated soil to below 50 m^{-3} (hypoxic) in waterlogged soil (Turner and Patrick, 1968) and sometimes the complete absence of oxygen (anoxic) due to microbial and respiratory activities (Ponnamperuma, 1984). Complete O_2 absence or reduction in its availability can restrict the aerobic respiration and provides an explanation for the reduction in the synthesis of ATP, particularly in submerged cells (Grichko and Glick, 2001; Greenway and Gibbs, 2003). In the sodic soils, pore spaces reduce as a result of a breakdown in soil structure after a quick addition of water, consequently reducing movement and availability of oxygen to plant roots (Grieve et al., 1986; Hillel, 2012).

2.1.2 Elemental and Metabolite toxicity:

Soil elemental toxicity is one of the main factors affecting plant performance under waterlogged and flooded conditions (Marschner, 1995; Shabala, 2011; Huang et al., 2015). The bioavailability of chemical elements in soil depends on different interacting factors such as

parent material, nature of organic matter, pH, redox potential, iron and manganese oxides, microbial activity and cation exchange capacity (Alloway, 2013; Shabala et al., 2014). Under waterlogged conditions, redox potential is mainly linked with the concentration of oxygen availability in soil. The redox potential declines quickly as soon as oxygen is depleted (Gambrell et al., 1991; Phukan et al., 2016). The flooded soils have significantly lower redox potential compared with well aerated upland soils (+350 and -250 mV, respectively) (Moldrup et al., 2013). O_2 depletion and decline in soil redox potential can occur within an hour due to nonstop consumption of oxygen by different aerobic organisms and plant roots (Balakhnina et al., 2010; Shabala, 2011). O_2 is the main electron acceptor and, after free O_2 depletion in soil, nitrate becomes the second electron acceptor after O_2. In the absence of O_2, microorganisms start using nitrate as an electron acceptor in the respiration process. Nitrate is reduced to nitrite (NO_2^-), various nitrous oxides (N_2O, NO) and in some cases into molecular nitrogen (N_2) in a denitrification process (Gambrell et al., 1991). On the other hand, nitrate reduction to NH_4^+ is less important when other electron acceptors such as Mn^{4+} and Fe^{3+} are accessible in sufficient amount (Ishii et al., 2011). Manganese oxides are the next electron acceptor followed by iron oxides used by anaerobic microorganisms. The solubility of these redox metals is enhanced under waterlogging conditions, attaining the level of concentrations considered to be toxic. Mn (IV) reduced to Mn (II) is followed by the reduction of ferric (Fe III) to ferrous (Fe II) iron (Mansfeldt, 2003). The reduction of iron and manganese oxides can also support the release of other non–redox-sensitive elements like Co, P, Ni, Cd and Zn (Li et al., 2012). As the redox potential decreases below -150 mV, SO_4^- is reduced to H_2S which is also toxic to plants (Frohne et al., 2011). Accumulation of toxic metals becomes more important for consumers health in paddy rice fields where the arsenic (As) availability to plants is increased in flooded soils (Shabala, 2011; Seyfferth et al., 2014).

Adverse physiological effects of secondary metabolites on plants have also been a focus of widespread exploration in previous studies. Plant phenolics are a leading chemical group concerned in allelopathy (Harper and Lynch, 1982; Siqueira et al., 1991; Fitzgerald et al., 1992). Phenolic compounds are the second abundant compounds in plants after carbohydrates (Bertin et al., 2003). The total concentration of phenolics normally differs between 100 and 500 mg kg^{-1} dry matter (Glass, 1973; Wu et al., 2001). In waterlogged soils, when pH inclines toward neutrality, the pH of rhizosphere would be lower than in bulk soil (Armstrong and Armstrong, 2001; Kirk, 2004). Under these circumstances, very low concentrations of organic

acids 1 mM or even less can become harmful for normal plant growth (Drew and Lynch, 1980; Armstrong and Armstrong, 2001; Wu et al., 2001).

2.1.3 Oxidative stress

The generation of reactive oxygen species (ROS) is a distinctive characteristic of hypoxic stress. Hydrogen peroxide (H_2O_2) and superoxide are two important radicals produced in a series of cellular reactions (Blokhina et al., 2003; Edmondson, 2014). H_2O_2 and superoxide are extremely reactive and may cause considerable injury to lipids, carbohydrates, proteins and nucleic acids. The quantity and formation of ROS are controlled by a large number of antioxidants (Noctor and Foyer, 1998; Bose et al., 2014). ROS formation in mitochondria, or in other compartments of the cell, may cause severe damage to mitochondrial components and initiate the degradation process (Cadenas and Davies, 2000). ROS levels are elevated under many abiotic stresses, compared with control conditions (Miller et al., 2008). To overcome ROS-induced damage, ROS scavenging systems are established in plants. For example, wheat grown under waterlogging conditions showed significantly higher expression of antioxidant enzymes than in control plants (Wong et al., 2004). However, experiments showed that an upregulation in antioxidant enzymes cannot always result in antioxidant defence (Blokhina et al., 2003), and a decrease in activity of waterlogging-induced antioxidant enzymes has also been reported (Sairam et al., 2009; Ashraf, 2012).

2.2 Signalling and adaptation to waterlogging stress

2.2.1 Anatomical adaptation

(1) Aerenchyma development:

Aerenchyma tissues are composed of gas films/channels which reduce the resistance of internal oxygen transportation from shoot to submerged parts of the plant (Jackson and Armstrong, 1999). For most species, the internal supply of oxygen is linked to the survival of plants under waterlogged conditions. Aerenchyma formation is one of the most important characteristics of waterlogging tolerance (Evans, 2004; Abiko et al., 2012). Aerenchyma may develop in existing root and shoot tissues, secondary tissues and/or newly formed organs. Aerenchyma has several types: lysigenous, schizogenous and expansigenous. It is called lysigenous aerenchyma if it is formed through programmed cell death (PCD) in the root cortex; schizogenous aerenchyma if

it is formed as a result of the separation of previously associated cells; and expansigenous aerenchyma if it is formed due to cell division and development without separation (Colmer, 2003; Evans, 2004; Seago et al., 2005; Steffens et al., 2011). The gaseous hormone ethylene stimulates the formation of aerenchyma by accumulating in plant organs under waterlogged conditions due to reduced diffusion rate (Laanbroek, 2010; Rajhi et al., 2011; Steffens et al., 2011). This notion has been proven by applying exogenous ethylene which induced aerenchyma formation rate while application of ethylene inhibitors intimidated the aerenchyma production (Drew et al., 1981; Jackson et al., 1985). However, ethylene is not always required in aerenchyma formation (Mommer et al., 2006; Parlanti et al., 2011). Aerenchyma development permits roots to put up with oxidative phosphorylation thus leading to higher ATP concentrations (Drew et al., 1985). Positive correlations were also reported between aerenchyma formation and better plant performance under waterlogged conditions when tested in a large number of wheat lines (Huang et al., 1994; Setter and Waters, 2003). Aerenchyma is not helpful in all cases in flooded environments. Plants may have to trade-off between mechanical strength, root porosity, and radial nutrient transport (Striker et al., 2007; Hu et al., 2014).

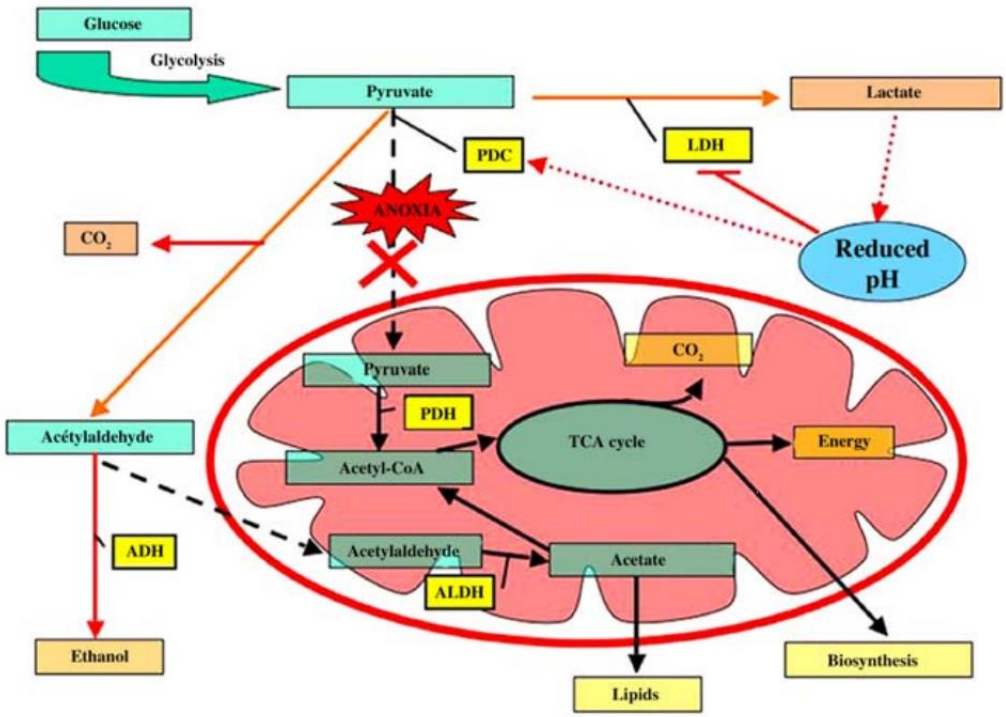

Fig. 2.1 Diagram of pH-stat hypothesis under aerobic respiration and during hypoxia. This diagram taken from Dat et al., 2004, Plant Physiology and Biochemistry 42: 273-282.

(2) Adventitious roots

Under waterlogged conditions, plants face a severe O_2 shortage, particularly submerged parts (roots). Most plant species can survive during a short-term episode of flooding by switching from aerobic respiration to glycolysis and fermentation for energy production (Bailey-Serres et al., 2012). Under long-term O_2 deficient conditions, plants (wetland species) display morphological adaptations such as adventurous root development (Fukao and Bailey-Serres, 2004; Voesenek et al., 2004). New adventitious roots are formed from the stem under waterlogged conditions to restore the normal root functioning. Deepwater rice can develop these roots in a normal developmental procedure (Vartapetian and Jackson, 1997; Lorbiecke and Sauter, 1999; Mergemann and Sauter, 2000). The development of adventitious roots has been shown to play a very important role in water and nutrient uptake from the upper surface of the soil (Visser and Voesenek, 2005; Sauter, 2013; Steffens and Rasmussen, 2016) and is considered as the leading trait contributing to flood tolerance at the morphological level (Justin and Armstrong, 1987; Seago et al., 2005; Colmer and Voesenek, 2009; Manzur et al., 2015). Normally, in flood-tolerant plants, adventitious roots can develop within days (Blom et al., 1994; Blom and Voesenek, 1996) but their development rate slows down with plant age (Mergemann and Sauter, 2000). Accumulation of ethylene and auxin in stem tissues play an important role in the emergence and development of adventitious roots in several dicots (Visser and Voesenek, 2005; Vidoz et al., 2010) and in monocots (Steffens et al., 2006). Application of ethylene precursors, 2- chloroethyl phosphonic acid (ethephon) and ACC, induces adventitious root development at the node of stem sections (Lorbiecke and Sauter, 1999). It appears that ethylene facilitates the death of epidermal cells around adventitious root initials, which accelerates the penetration of the roots throughout the submergence (Mergemann and Sauter, 2000; Steffens and Sauter, 2005). The important roles of adventitious roots also include supplying hormones, minerals and acting as descends for shoot integrates and metabolites (Armstrong et al., 1994).

(3) ROL control

Most of the wetland species use the basal root zones to avoid inevitable oxygen loss by forming a complete or partial barrier to radial oxygen loss (ROL) in their epidermis, exodermis and/or sub-epidermal layers (Jackson and Armstrong, 1999; Visser et al., 2000; McDonald et al., 2002; Colmer, 2003; Abiko et al., 2012; Manzur et al., 2015). The origin of the signal for a barrier stimulation is still unclear but the barrier to ROL is considered as an adaptive trait of

many wetland plants (Kotula et al., 2014; Phukan et al., 2016). By reducing the losses of oxygen to the rhizosphere, longitudinal oxygen diffusion in the aerenchyma is increased in the direction of apex due to the barrier to ROL in the basal part of root (Voesenek et al., 1999; Armstrong et al., 2000; Visser et al., 2000; Colmer, 2002). The thick laterals with overlying rhizospheres and a low rate of ROL could assist an oxidized zone to persevere even when soil microorganisms consume oxygen. ROL could defend the apex and adventitious roots by reducing soil toxins, whereas in mature root zones it was suggested physically preventing the entry of soil toxins (Armstrong, 1979; Shiono et al., 2010; Watanabe et al., 2013). However, in some situations, the physical barrier to ROL may inhibit normal root functions. It was supposed that it can block water and nutrients uptake by roots in wetland species (Armstrong, 1979; Končalová, 1990). Sometimes, very low concentrations of organic acids can also trigger a barrier development in roots. Due to the accumulation of potentially toxic organic compounds, root growth and K^+ content decline in the wetland species *H. marinum* (Kotula et al., 2014). Most of the dryland species such as barley, wheat, rape, oats and sorghum lack this feature of ROL development in contrast to wetland species (Colmer, 2003; Abiko et al., 2012; Yamauchi et al., 2013). The significance of a ROL barrier for waterlogging tolerance was confirmed by the introduction of a barrier from a wild relative (*Hordeum marinum*) into wheat (*T. aestivum*) (Malik et al., 2011).

2.2.2 Physiological and biochemical adaptation

(1) Metabolic shifts

The oxygen plays an important role in the energy providing pathways of plant cells, regulation of metabolic activity and energy production (Dennis et al., 2000; Geigenberger, 2003). Oxygen is also essential for some vital cellular pathways, including sterol, haemoglobin and fatty-acid biosynthesis (Geigenberger, 2003). However, plants lack an efficient system for oxygen transport compared to animals (Dennis et al., 2000). Under hypoxia or anoxia, the plant tissues suffer from energy disaster (Gibbs and Greenway, 2003) in both waterlogging tolerant and sensitive plants due to reduced root respiration (Marshall et al., 1973; Lambers, 1976; Drew, 1983, 1990; Hossain and Uddin, 2011). Being a terminal acceptor of electrons in oxidative phosphorylation, the presence of oxygen in aerobic organisms is a requirement for efficient production of ATP (Bailey-Serres and Chang, 2005; Voesenek et al., 2006). The gas diffusion rate is slower in solution compared to the air (Ponnamperuma, 1984; Colmer, 2003). Oxygen

deficiency creates a major energy disaster due to rapid depletion in flooded soils (Bailey-Serres and Voesenek, 2008; Licausi and Perata, 2009). For example, maize roots showed a two-fold reduction in relative ATP contents after anoxia treatment (Brauer et al., 1997). In another study, a two-fold decrease in H^+-ATPase activity and 80% reduction in ATP contents were reported in soybean roots after 12 h of anoxia stress (Shen et al., 2006). The metabolic adaptations to oxygen deficient conditions are; maintenance of carbohydrate supply for anaerobic respiration, prevention of cytoplasmic acidification and developing efficient anti-oxidative defence system (Davies, 1980; Armstrong et al., 1994; Setter et al., 1997).

(2) Control of ionic homeostasis

Mineral nutrition is crucial in plants in the context of adaptive responses to waterlogging stress. Plant roots are very sensitive to oxygen deprivation, reflecting the changed pattern of ionic homeostasis under flooding stress (Drew, 1988; Subbaiah and Sachs, 2003). Continues supply of oxygen is crucial for respiration and the ATP generation. Anaerobic metabolism cannot maintain the level of energy required for efficient ion movement and several other vital processes in plants (Armstrong and Drew, 2002). Many studies showed a significant decline in the concentrations of N, P and K in plant roots and their transportation to shoots under waterlogging stress (Trought and Drew, 1980, 1980; Buwalda et al., 1988; Drew, 1988; Boem et al., 1996; Singh et al., 2002). Nitrogen uptake and transport to shoots were shown to be reduced in barley seedlings within two days (Drew and Sisworo, 1977). Mg^{2+} and Ca^{2+} transport are not closely linked to energy metabolism as their contents in plant shoots are always less affected by oxygen deprivation in flooding conditions compared to N, P and K (Drew and Sisworo, 1979; Cannell et al., 1980; Trought and Drew, 1980; Stieger and Feller, 1994). In the case of chlorosis and nitrogen deficiency, plants can use the altered source of nutrients sink as shoots can also influence the redistribution of mobile nutrients from old leaves to new growth sites (Boem et al., 1996). Nitrogen compounds can be remobilized very rapidly in barley plants, nitrogen transferred within two days from older leaves to younger leaves and tillers (Drew and Sisworo, 1979). In barley plants, as an early response to waterlogging stress, the redistribution of phosphorus and potassium also takes place with these minerals being translocated from older leaves to younger in the situations when plants are unable to supply nutrients from roots (Drew and Sisworo, 1979; Trought and Drew, 1980). Waterlogging stress favours the availability of exchangeable Fe and Mn (Sharma and Swarup, 1989; Stieger and

Feller, 1994) and unusually large accumulations of these metals are associated with toxicity symptoms (Drew and Lynch, 1980).

(3) ROS detoxification

Antioxidants have long been recognised as significant adaptive traits during oxidative stress. The positive role of antioxidant enzymes has been reported in various studies underlying flooding tolerance (Monk et al., 1987; DREW, 1992; Noctor and Foyer, 1998; Boo and Jung, 1999; Smirnoff, 2000; Blokhina et al., 2003). The complex antioxidant network is controlled at the site of synthesis and through interaction with ROS (Blokhina and Fagerstedt, 2010). The plant antioxidant enzyme defence system is characterized by a number of molecules having diverse chemical nature and functioning. Antioxidant enzymes are capable of detoxification of ROS in different parts of living tissues of plants. Plants have limited molecular antioxidant resources including tocopherols, ascorbate, glutathione, and carotenoids (Pietta, 2000; Hernández et al., 2009; Blokhina and Fagerstedt, 2010). For the regeneration of antioxidants in dynamic forms and to sustain the redox status a collection of enzymes plays a crucial role in the support of antioxidant defence systems such as thioredoxin (TRX) reductase, dehydroascorbate reductase (DHAR), lipoamide dehydrogenase, glutathione (GSH) reductase and thioltransferase (glutaredoxin) (Blokhina and Fagerstedt, 2010). The effectiveness of the antioxidant systems is amplified by direct enzymatic removal of toxic ROS by superoxide catalases (CAT), dismutases (SODs) and peroxidases (PRX) (Blokhina and Fagerstedt, 2010).

(4) Dealing with elemental toxicities

Plants ability to deal with toxic elements like Fe, Mn and Al is considered an important adaptive trait for plant performance and survival both in acidic soils under waterlogging and well-drained conditions (Khabaz-Saberi et al., 2012; Herzog et al., 2016). A better ability of Fe extrusion from plant shoots is casually linked with Fe tolerance in wheat (Khabaz-Saberi and Rengel, 2010) and rice (Audebert and Sahrawat, 2000). There is more than one mechanism involved in Fe tolerance such as Fe uptake through roots, prevention of Fe transport to shoots, Fe xylem loading and unloading and sequestration of Fe in vacuoles (Palmer and Guerinot, 2009; Conte and Walker, 2011; Shabala, 2011). Aerenchyma facilitates oxygen availability in the root zone which further contributes to the Fe oxidation from Fe (II) to Fe (III) in flood tolerant species (Asch et al., 2005; Mongon et al., 2014; Rout and Sahoo, 2015). The ability to exclude reduced toxins in mangroves and wetland species increase their tolerance against

elemental toxicity (Becker and Asch, 2005; Sahrawat, 2005). Fe(III) precipitation in the aerobic apoplast may help to avoid oxidative stress, but at the same time, this mechanism also reduces the uptake of other important macro and micronutrients (Nishiuchi et al., 2012; Maathuis and Diatloff, 2013). Phytoferritin (an iron storage protein) formation is another mechanism to restrain surplus Fe and avoid the building up of toxic Fe^{2+} (Arnaud et al., 2006; Yadav, 2011). Recently YSL4 and YSL6 have been reported as an iron transporter, which is responsible for Fe extrusion from plastids and their double mutant after knocking out shows a very high sensitive response to excess Fe (Divol et al., 2013). Removal of Mn in the oxidized form at the root surface and in the shoot apoplast and control of free Mn^{2+} in the apoplast, are also features contributing to Mn tolerance (Iwasaki et al., 2002). Shoot cell walls and epidermal cells play a major role in the avoidance of excess Mn build-up in the photosynthetic parts that are further verified by the results in which silicon removed the toxic effects of Mn in plants (Iwasaki et al., 2002; Ma, 2004).

2.2.3 Waterlogging stress signalling

(1) Ethylene signalling

Ethylene is an important gas required for numerous developmental processes in plant systems. Ethylene also performs as an important signalling molecule in an unfavourable environment particularly under oxygen-limited conditions in higher plants. Due to gaseous nature, it is difficult for ethylene to leave plant so it may accumulate easily in plants under flooding conditions (Adams-Phillips et al., 2004; Abeles et al., 2012; Voesenek and Bailey-Serres, 2015; Loreti et al., 2016). In a study, it was revealed that the ethylene is perceived by a group of five receptors which is bound to membranes of Golgi and the endoplasmic reticulum (ER) in Arabidopsis (Kendrick and Chang, 2008). The ethylene receptors are insensitive controllers of its signalling that is similar in sequence and structure with bacterial two-component histidine kinase (Vaidya et al., 1998; Binder et al., 2004). Receptors form dimers which bind ethylene by a copper cofactor at the N-terminal membrane-spanning area of the protein, and this ethylene binding inactivates receptors, consequently ethylene accomplishment (Hua et al., 1998; Chen et al., 2010; Voesenek and Sasidharan, 2013). In the next step receptors physically interrelate with a positive ethylene insensitive 2 (EIN2) and a negative constitutive triple response (CTR1) regulators of ethylene signalling. It is suggested that, in the absence of ethylene, the CTR1 is linked with the receptors in such a manner that signalling to EIN2 is

stopped which is a further downstream target. After the ethylene binding, slow changes in the structure of CTR1 letting EIN2 to interact with the kinase part of the receptors. EIN2 activation induces the build-up of the transcription factors (TFs) such as ethylene insensitive 3 (EIN3) and ethylene insensitive 3-like 1 (EIL1); these are enough to influence the ethylene response factors (ERFs) which are responsible for the activation of ethylene target genes (Chao et al., 1997; Nakano et al., 2006; Voesenek and Sasidharan, 2013). Essentially, a small proportion of only 10% of the genes belonging to the ERF family is controlled through ethylene signalling (Nakano et al., 2006; Pré et al., 2008)

(2) pH signalling

A fast and distinctive response to low oxygen (hypoxia/anoxia) is the rapid decline of cytosolic pH. In most cases, pH drops by around half a unit but can drop further in hypoxia-sensitive species. This decline in pH has been discussed as the second messenger (Felle, 1989; Blatt, 1992; Subbaiah and Sachs, 2003; Voesenek et al., 2006) and/or stress signal (Wilkinson, 1999; Felle, 2001; Dat et al., 2004; Felle, 2005). Cytosolic acidification results from the low activity of proton pumps due to the limited supply of ATP under hypoxic conditions and also through proton release from ATP hydrolysis. Furthermore, the reduction in pH of cytosol inhibits the production of lactate dehydrogenase (LDH) but favours the pyruvate decarboxylase (PDC), thereby converting pyruvate to acetaldehyde which then is converted to ethanol by alcoholic dehydrogenase (ADH) enzymes as it works better at low pH (Armstrong et al., 1994; Dennis et al., 2000). Interestingly, lactate production always remains low in rice seedlings, which are hypoxia-tolerant species (Menegus et al., 1991; Perata et al., 1992; Dat et al., 2004). In addition, these changes in cytosolic pH have been linked to abscisic acid (ABA) in regulating stomata and considered to be a signal during hypoxia stress (Wilkinson, 1999).

(3) Ca^{2+} signalling

Ca^{2+} plays a crucial role in plants as a second messenger in signal transduction. Several studies showed that Ca^{2+} responds to almost every biotic and abiotic stress signals (Miller et al., 2008; McAinsh and Pittman, 2009; DeFalco et al., 2010) and causes changes in the cellular Ca^{2+}, mainly in the cytosol and in some cases in the nucleus and other organelles (Pozzan et al., 1994; Chin and Means, 2000; Hepler, 2005; Reddy et al., 2011). Low oxygen stress rapidly elevates cytosolic Ca^{2+} in the cells of wheat, maize, rice and Arabidopsis (Yemelyanov et al., 2011; Lindberg et al., 2012; Wang et al., 2016) and this Ca^{2+} elevation is essential for gene expression

and acclimation responses at tissue, cellular and whole plant levels (Shabala et al., 2014; Tran et al., 2017; Wang et al., 2017). The mitochondrion is the primary site for oxygen consumption; it is also believed that it may serve as a Ca^{2+} store under hypoxia stress in maize. Tissue-specific studies also showed that Ca^{2+} signals originate most likely from mitochondria as observed by confocal analysis (Subbaiah et al., 1998). Hypoxia stress depolarizes mitochondrial membrane and induces Ca^{2+} release from mitochondria to cytosol. At the same time, K^{+} channels are inhibited due to plasma membrane depolarization caused by hypoxia, leading to significant Ca^{2+} influx into the cytosol (Fähling, 2009; Yemelyanov et al., 2011; Lindberg et al., 2012). These Ca^{2+} responses/signals need to be interpreted, transmitted and amplified by Ca^{2+}-binding proteins, also known as Ca^{2+} sensors. The most common Ca^{2+} sensors are Ca^{2+}-dependent protein kinases (CDPKs), calmodulin (CaM), calmodulin-like proteins (CMLs), some DNA binding proteins and a few enzymes. These sensors play a vital role in Ca^{2+} signalling during plant adaptive responses under hypoxia stress (Harper and Harmon, 2005; Dodd et al., 2010; Reddy et al., 2011; Whalley et al., 2011; Ranty et al., 2016).

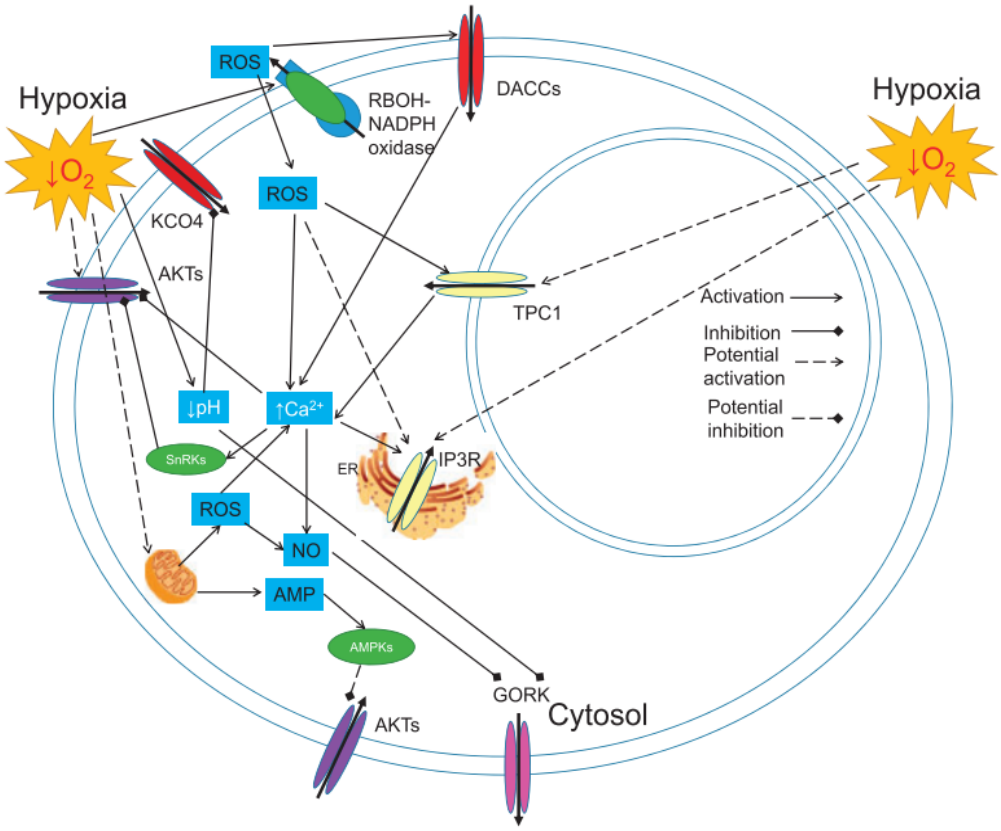

Fig. 2.2 An anticipated model for ion channels in hypoxia sensing in plant cells. This model was taken from a recently published review by Wang et al., 2017, Plant and Cell Physiology 58:1126-1142.

(4) K^+ signalling

There is emerging evidence for the role of K^+ in signalling in addition to its well-established position as important macro-nutrients of plants (Amtmann et al., 2005; Ashley et al., 2005; Cakmak, 2005; Wang and Wu, 2013; Anschütz et al., 2014). K^+ also is considered as an essential element for acclimation during adaptive responses of plants under oxygen-limited conditions (Mugnai et al., 2011; Shabala et al., 2014). Indeed, a large net K^+ leak is observed in root tissues treated with hypoxia solution (Zeng et al., 2014; Gill et al., 2017). The adaptive significance of this K^+ leakage is multi-fold. In the short term, a continuous decrease in the cytosolic K^+ pool provides a mean for plant to shut-down several energy expensive metabolic processes such as protein synthesis and direct saved energy to defence-related processes that may help to avoid energy crises (Dreyer and Uozumi, 2011; Shabala, 2011; Pottosin and Shabala, 2014). In the long term, a transient reduction in the cytosolic K^+ pool may contribute in the programmed cell death (PCD) by activating different catabolic enzymes like proteases (Shabala et al., 2007; Demidchik et al., 2010). This PCD may reflect an adaptive trait if it takes place in the mature root cortex after removing some cells to form aerenchyma; which enhance the porosity and ultimately supports the efficient oxygen transport under waterlogged conditions (Shabala et al., 2014; Zeng et al., 2014). Moreover, this process of aerenchyma formation is supplementary to (Visser and Voesenek, 2005; Huber et al., 2008) or self-directed of (Shabala, 2011), ethylene-facilitated pathways. Conversely, if the same process of PCD occurs in the root meristem (which is not capable of forming aerenchyma), this may result in a complete halt of growth and the ultimate death of the root apex (Gibbs and Greenway, 2003; Jackson and Drew, 2012; Shabala et al., 2014). At the same time, long-term K^+ loss in the mature zone may also be detrimental to the operation of over 70 enzymes regulated by K^+ (Dreyer and Uozumi, 2011).

(5) ROS signalling

Generation of ROS is typical for every living tissue under longer periods of oxygen deprivation that is more severe after re-oxygenation (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009; Blokhina and Fagerstedt, 2010). Mitochondria, due to their nature, are very sensitive to fluctuations in the levels of oxygen and the ROS formation are most profound under hypoxia stress (Klok et al., 2002; Mansfield et al., 2005; Sena and Chandel, 2012). The other site for ROS accumulation via NADPH oxidases is in the apoplast. A recent study showed the highest ROS accumulation being after 12 h that was accompanied by significant up-

regulation of NADPH oxidases and MnSOD along with a reduction in catalase expression (Xu et al., 2013). On the other hand, knocking out *RBOHD* reduced the production and accumulation of both superoxide and H_2O_2 (Torres et al., 2005; Wang et al., 2016). Furthermore, the signalling importance of ROS has been established when the transcript levels of a respiratory burst oxidase was increased under hypoxia stress (Klok et al., 2002). Hypoxia-induced upregulation of RbohA has also been reported in *Medicago truncatula* (Marino et al., 2011; Andrio et al., 2013). Under hypoxia stress, ROS produced in mitochondria activate the mitogen-activated protein kinases (MPK3, MPK4, MPK6) in seedlings of Arabidopsis seedlings (Chang et al., 2012) and H_2O_2 produced in mitochondria could enter the cytosol after crossing mitochondrial membrane (Hamanaka and Chandel, 2009). ROS signalling also plays an integral role in the anatomical adaptations to low oxygen stress by triggering the process of aerenchyma formation. A recent study showed the requirement for elevated ROS for PCD during the development of adventitious roots in seedlings of rice (Steffens et al., 2012).

2.3 Role of membrane transporters in waterlogging stress tolerance

2.3.1 Oxygen availability and its impact on ionic homeostasis

Oxygen availability to plant roots reduces under waterlogged conditions and impacts adversely root metabolism and ionic homeostasis. The oxygen availability and requirement in plant roots is highly tissue/cell-specific. In addition, the duration of anoxic/hypoxic stress is also an important contributing factor for oxygen availability (Armstrong and Drew, 2002; Mustroph et al., 2010). The oxygen uptake and requirement was shown to be significantly higher in the functionally more active root apex zone under aerated conditions (Mancuso and Boselli, 2002; Colmer, 2003; Pang et al., 2006). Additionally, the respiration rate and ATP content were two-fold higher in the apex than in the rest of the barley root (Reid et al., 1985). After hypoxia treatment, the uptake rate of oxygen was reduced rapidly in the elongation zone and in the mature zone the uptake has changed into net oxygen efflux. The oxygen depletion from both the root zones was highly time-dependent with more oxygen being lost as the hypoxia stress progressed (Pang et al., 2006; Zeng et al., 2014). Under the long-term oxygen-deprived conditions, the root zone behind the apex where cells are fully expanded was less affected due to continues supply of oxygen from leaves after the development of ethylene-dependent gas-filled channels (aerenchyma) whereas the apical zone that lacks the property of aerenchyma development also suffers the most due to oxygen shortage (McDonald et al., 2001; Aguilar et

al., 2003; Dat et al., 2006; Herzog et al., 2016). For example, net losses of K^+ and Cl^- were significantly higher and faster in the apical part compared to mature root under oxygen-limited conditions (Greenway et al., 1992). The internal concentration of oxygen supply via aerenchyma was reduced in a curvilinear slope with a distance towards root tip (Armstrong et al., 2000; Armstrong et al., 2008; Verboven et al., 2014). Similarly, oxygen gradients have also been found along radial root profiles under hypoxia stress (Armstrong et al., 1994; Gibbs et al., 1998). The excised maize roots showed a steep O_2 concentration decline from the outer layers of cells to the stelar cells under oxygen-limited conditions (Gibbs et al., 1998), showing more accumulation of anaerobic metabolism products (typically alanine, ethanol) and elevated events of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (ADH). Finally, the apical and stele regions of root receive a compromised oxygen supply as both occurred at the ends of longitudinal and radial diffusion paths (Armstrong and Beckett, 1987; Gibbs et al., 1998; Bramley, 2006; Colmer and Greenway, 2010). Under oxygen-deprived conditions, the restricted oxygen availability in the apical zone and stele of roots reduced the H^+-ATPases activity, so the trans-membrane H^+ difference could be diminished and finally, plants were compromised on nutrients uptake, xylem loading, and maintenance of intracellular ionic homeostasis.

2.3.2 H^+-pump activity and membrane potential maintenance

The proton-pumps ATPase (H^+-ATPase) are the main electrogenic systems responsible for maintaining negative membrane potential at the plant plasma membrane and contribute in nutrient uptake through roots (Sondergaard et al., 2004; Teakle et al., 2013; Shabala et al., 2014). At the same time, H^+ pumps are major consumers of ATP. A continuous supply of oxygen is very important in the process of efficient production of ATP in aerobic organisms (Voesenek et al., 2006; Wegner, 2010). In waterlogged soils, oxygen deficiency rapidly depolarizes plasma membrane potential of waterlogging-sensitive species of higher plants (Buwalda et al., 1988; Teakle et al., 2013; Zeng et al., 2014). Also, reduced oxygen availability for ATP production leads to energy crises which results in a limited supply of energy to fuel H^+-ATPase pumps which enable H^+ pumping out of the cell (Bailey-Serres and Voesenek, 2008; Licausi and Perata, 2009). As a result, the channel-mediated uptake of many essential solutes is reduced, as a consequence of a reduced driving force for H^+ - coupled symport systems (Glass and Fernando, 1992; Kreuzwieser and Gessler, 2010; Wang et al., 2013). It was

shown that the H^+-ATPase activity was significantly reduced after 2 h of anoxic treatment in pea epicotyls (Koizumi et al., 2011).

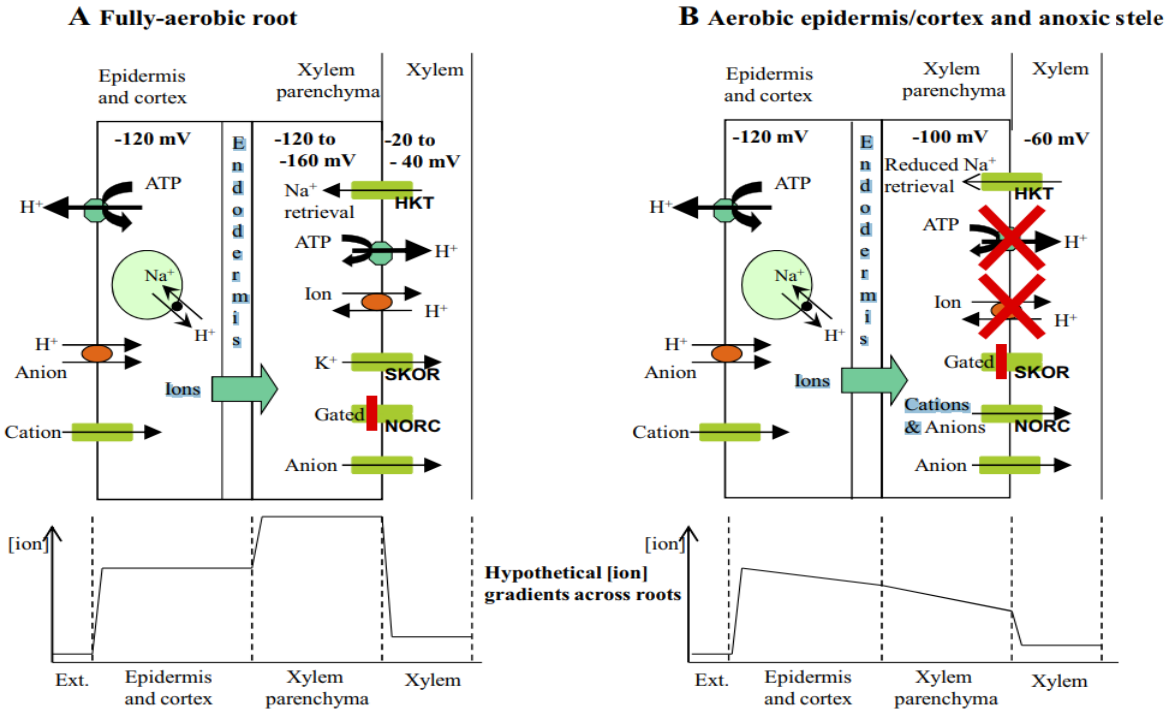

Fig. 2.3 This diagram of ion transport was taken from Colmer et al., 2010, Journal of Experimental Botany 62: 39-57.

The two other categories of H^+ pumps are the tonoplast-located vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-inorganic pyrophosphatase (V-PPase). These pumps show important roles for the accumulation of key ions into the vacuoles by generating electrochemical H^+ gradients for solute transport through vacuolar membranes (Sze et al., 1999; Sondergaard et al., 2004; Beyenbach and Wieczorek, 2006). V-ATPase pump re-exports the H^+ and helps to counter the cytosolic acidification under oxygen-limited conditions (Gibbs and Greenway, 2003; Dreyer and Uozumi, 2011). It can be activated/deactivated within a few seconds to minutes by changing the availability of oxygen. Reduced oxygen supply massively inhibits the plasma membrane (PM) and V-H^+-ATPase activities (Zhao et al., 2012). In the state of an inadequate V-ATPase activity, the vacuolar H^+-pyrophosphatase (V-PPase) performs as an additional or alternative powerhouse of the tonoplast (Greenway and Gibbs, 2003; Fan et al., 2017). It was recommended that a shift from V-ATPase to V-PPase-driven H^+ pumping is useful to roots when oxygen availability is reduced (Greenway and Gibbs, 2003). The breakdown of PPi is also favourable to ATP levels when ATP availability and cytoplasmic pH drop down due to reduced availability of oxygen (Felle, 2005).

2.3.3 Control of intracellular K^+ homeostasis

K^+ is the second most abundant mineral element in plant tissues contributing almost 2% to 10 % of the total plant dry matter (Leigh and Wyn Jones, 1984; Marschner, 2011). The availability of K^+ depends on different soil types and several other environmental factors. Generally, the available concentration of K^+ ranges from ~0.025mM to 5mM in soil (Mc Lean and Watson, 1985; Maathuis, 2009), but the plants managed to keep cytosolic K^+ concentrations in tissues at a level of ~150 mM (Leigh and Wyn Jones, 1984; Kronzucker et al., 2008). The availability of K^+ to plant tissues at a persistent level is important to enable several essential roles in plants such as cell turgor pressure maintenance, ionic homeostasis, phloem loading and solute transport, and cytosolic pH regulation. (Dreyer and Uozumi, 2011; Nieves-Cordones et al., 2014; Shabala and Pottosin, 2014; Zörb et al., 2014). The plant's ability to take up K^+ is mostly reduced under O_2 limited conditions (Zhao et al., 2012; Zeng et al., 2014), but detrimental effects of waterlogging on plants may be alleviated by the extracellular application of K^+ to soil or as a foliar spray (Römheld and Kirkby, 2010; Ashraf et al., 2011).

Under long-term oxygen-deprived conditions, a significant decline in the shoot K^+ content was reported in several species (e.g. eucalypts - Close and Davidson, 2003; *Hordeum marinum* - Malik *et al.*, 2008; wheat - Trought and Drew, 1980; lucerne - Smethurst *et al.*, 2005; soybean - Board, 2008). More severe and prolonged anoxia stress resulted in a significant K^+ leakage from the plant's root tissues (Morard et al., 2004; Kırmızı and Bell, 2012). In a recent study, Teakle et al. (2013) used two halophyte grasses (*Puccinellia ciliata* and *Thinopyrum ponticum*) differing in WL stress tolerance. The WL-tolerant *P. ciliata* showed significant uptake of K^+ under oxygen-deprived conditions, while more sensitive *T. poticum* showed a substantial K^+ leakage when examined by the microelectrode ion flux estimation (MIFE) system. Similar results were also reported under hypoxia stress when two WL-tolerant cultivars CM72 and TX9425 showed higher uptake of K^+ in the root mature zone after hypoxic treatment compared to Japanese WL-sensitive cultivar Naso NIjo. Likewise, the overall magnitude of K^+ leakage from the root elongation zone was higher in sensitive than tolerant cultivars (Pang et al., 2006; Zeng et al., 2014). The above-reported controversies may be explained by putting both transport and regulation of K^+ homeostasis under O_2 limited conditions into the context of the root tissue- and genotypic- specific manner.

Under oxygen deficient conditions, the net K^+ loss may be triggered by several factors such as channel opening due to membrane depolarization, changed K^+ selectivity to non-selective

membrane- and ROS-activated channels (Sharma et al., 2012; Shabala and Pottosin, 2014). Earlier pharmacological studies suggested that hypoxia-induced changes in K^+ fluxes could potentially be mediated by both voltage-dependent K^+-inward (KIR) and K^+-outward (KOR) rectifying channels (Shabala, 2003; Pang et al., 2006). However, strong depolarization of membrane potential under hypoxic conditions (Teakle et al., 2013; Zeng et al., 2014) may discourage the involvement of KIR channels (such as AKT or KAT) as thermodynamically impossible. This hypothesis should be validated in the direct experiments.

2.3.4 ROS production and impact on ionic homeostasis

Under oxygen-deprived conditions, ROS are synthesized in different intracellular compartments including apoplast, mitochondria, chloroplast and peroxisome sources (Blokhina and Fagerstedt, 2010; Wang et al., 2016). Apoplastic ROS production is mediated by plant respiratory burst oxidase homologues (RBOHs) proteins located at the plasma membrane (Suzuki et al., 2011; Baxter et al., 2013). NADPH oxidase is also a key contributor to ROS generation in flooded conditions (Sagi and Fluhr, 2006). RBOHs proteins oxidize NADPH with the production of ($O_2^{\bullet-}$) anions. Short-lived ($O_2^{\bullet-}$) anions may convert to (H_2O_2) naturally, or oxidized by ascorbate peroxidases (POD) or superoxide dismutase (SOD) at a later stage with the progression of oxidative stress (Sagi and Fluhr, 2006; Subbaiah, 2009). In a recent study, Arabidopsis showed a significant up-regulation in *RBOHD* expression after 6 h of anoxia treatment (Yang and Hong, 2015). At the same time, a rbohD knockout mutant showed a reduced accumulation of ($O_2^{\bullet-}$) and (H_2O_2) during recovery responses (Forman and Torres, 2002; Pucciariello et al., 2012). Under low oxygen, mitochondria are another main candidate for intracellular ROS production (Zorov et al., 2014). Mitochondria have been shown to generate ROS ($O_2^{\bullet-}$, H_2O_2) due to the electron release at the ubiquinone site (Gille and Nohl, 2001). As a result of the reduced availability of oxygen, H_2O_2 production and accumulation in Arabidopsis are reliant on ROP GTPase (Baxter-Burrell et al., 2002).

As mentioned earlier, the cellular ROS balance can be disturbed under stress conditions due to either enhanced production of ROS or reduced antioxidants activity in plants (Steffens, 2014). Under moderate stress conditions, ROS formation primarily acts as a regulatory mechanism such as signalling by activating defence and immunity reactions. However, when stress is severe, ROS generation overlays the oxidative stress additional to a given stress factor, damaging cellular components and causing their dysfunction. H_2O_2 plays a role in activating a range of cation-permeable non-selective cation channels (Mori and Schroeder, 2004;

Demidchik et al., 2007; Ordonez et al., 2014). Thus, affecting intracellular K^+ and Ca^{2+} homeostasis (Shabala and Pottosin, 2014) may initiate programmed cell death (PCD). In addition, by interacting with transition metals, either in the apoplast (Demidchik and Shabala; Demidchik et al., 2007) or in the cytosol (Rodrigo-Moreno et al., 2013), H_2O_2 can form hydroxyl radicals, that directly contribute to activation of GORK channels (Demidchik et al., 2014). On the other hand, ROS production contributes to aerenchyma formation by eliminating few cells from the root mature zone, further supporting a role in anatomical adaptation to waterlogging.

2.4 QTL mapping for waterlogging stress tolerance

2.4.1 QTL mapping as a breeding tool

Developing waterlogging tolerance in plants is one of the major objectives of breeding programs. Waterlogging stress tolerance estimation is a complex process because it could be affected by multiple factors such as duration of stress, the severity of stress and difference of genetic makeup in different plant species (Setter and Waters, 2003). Moreover, species respond differently to waterlogging stress, as shown for rice, maize, and barley (Pang et al., 2004). Genetic differences also exist for waterlogging tolerance between genotypes of the same species (Gardner and Flood, 1993; Colmer, 2002; Smethurst and Shabala, 2003; Setter et al., 2008; Khabaz-Saberi and Rengel, 2010; Zhou, 2010; Narsai et al., 2011; Zeng et al., 2013). For example, wheat showed significant genetic diversity for waterlogging stress tolerance when grown under hypoxia stress (Huang et al., 1994). Substantial genetic differences towards waterlogging tolerance based on several physiological indices during hypoxia stress and reoxygenation were shown in 14 wheat genotypes and DH lines (Setter et al., 1999).

Waterlogging tolerance in physiological studies is assessed by the survival or the maintenance of growth rates under waterlogging stress relative to well-drained conditions, whereas waterlogging tolerance from the agronomic point of view is the maintenance of relatively high grain yields under waterlogged conditions relative to control plants (Setter and Waters, 2003). To achieve waterlogging tolerance, several approaches were applied on the basis of a large number of morphological, physiological and biochemical changes. Producing waterlogging tolerant species may now be accelerated by using advanced genetic approaches including quantitative trait loci (QTL). Detection of QTLs in breeding programs provides more advanced information as these QTLs are of direct relevance to the development of tolerant

varieties (Mackill et al., 1999; Toenniessen et al., 2003). In the meantime, development of near-isogenic lines (NILs) based on the advancement of QTL mapping is important for targeting the waterlogging tolerant genes more precisely, which is fundamental to achieve the waterlogging tolerance goals. NILs can help to eliminate the noise triggered by supplementary genes or due to population structure and supports the accuracy of QTL position (Palstra and Ruzzante, 2008; Boopathi, 2012). Also, the genetic background of traits is crucial for precise phenotypic assessments (Rostoks et al., 2005; Wang et al., 2012). Additionally, the NIL-derived populations, permit the alteration of a quantitative trait into a Mendelian factor by segregating primarily for a targeted locus and hence, making possible the accurate location of a QTL (Ma et al., 2012).

To identify the QTLs associated with waterlogging tolerance in recent genetic studies, several plant responses and traits have been targeted under waterlogging stress including germination rate, total dry shoot and root weight, plant height, chlorophyll damage index, leaf chlorosis, survival, adventitious root development, aerenchyma formation, fast petiole extension, hyponastic growth, plant biomass indices, stomata closure, photosynthetic characteristics (Justin and Armstrong, 1987; Armstrong et al., 1994; Armstrong and Strange, 2001; Zhou et al., 2002; Cornelious et al., 2005; Mano et al., 2005; Striker et al., 2005; Mano et al., 2006; Mano et al., 2009; Xue et al., 2010; Zhou, 2011; Striker, 2012). Several QTLs associated with waterlogging tolerance in wheat, maize, barley, rice and soybean were also identified by targeting these traits, which open up new avenues for possible marker-assisted selection and discovery of underlying genes.

2.4.2 QTL mapping for anatomical traits

Among the targeted traits for waterlogging tolerance, aerenchyma formation and tight radial oxygen loss (ROL) are more directly related traits to plant's ability to survive under waterlogged conditions. Aerenchyma (gas films) formation in cortical cells improves the O_2 supply from shoots to roots under waterlogging stress. Due to diffusion gradient the concentration of O_2 declines with the distance from shoot base towards root apex (Gibbs et al., 1998; Yamauchi et al., 2013; Sasidharan and Voesenek, 2015). At the same time, roots of several wetland plant species can develop ROL barrier to control the O_2 loss from basal zone, and finally supports the longitudinal diffusion of O2 through aerenchyma towards the root tip (Armstrong et al., 2000; Soukup et al., 2007; Garthwaite et al., 2008). The direct measurement of these two traits may offer more advantages to achieve the goal of developing waterlogging

tolerant verities. For example, the ability to develop higher root porosity and ROL barrier in *Hordeum marinum* has been productively introduced in cultivated wheat to enhance waterlogging tolerance (Malik et al., 2011).

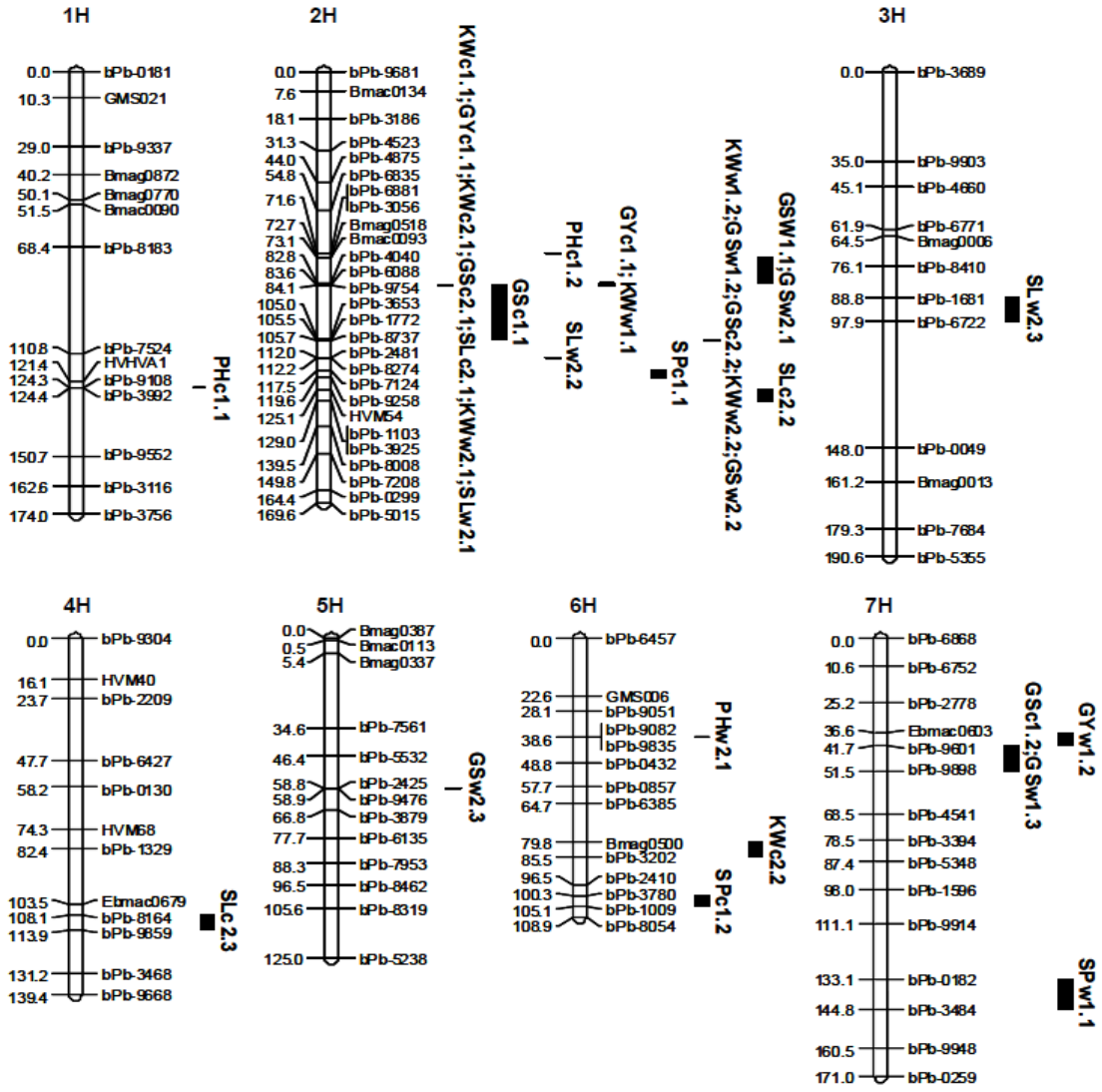

Fig. 2.4 QTL for agronomic traits identified on different chromosomes in barley. One QTL was detected for kernel weight on chromosome 2H. Two QTL were identified for grains per spike on chromosomes 2H, 5H and 7H. Three QTL for spikes per plant on chromosomes 2H, 6H and 7H, three QTL for plant height on chromosomes 1H, 2H and 6H and two QTL for grain yield on chromosomes 2H and 7H were also detected. This figure was taken from Xue et al., 2010, Journal of Zhejiang University Science B, 11: 169-176.

Interestingly, the ability to develop aerenchyma and plant performance under waterlogging stress have been positively correlated (Huang et al., 1994; Setter et al., 1999). In a study conducted by Pang et al. (2004) a waterlogging tolerant variety of barley TX9425 showed a higher percentage of aerenchyma in root cross-sections compared to relatively sensitive variety Naso Nijo. Over the years, significant numbers of QTLs related to the

development of aerenchyma were identified in several plant species for waterlogging stress tolerance (Mano et al., 2005; Mano and Omori, 2007; Broughton et al., 2015; Zhang et al., 2016). For example, in maize four QTLs for constitutive aerenchyma formation were identified in three different chromosomes (Mano et al., 2008; Mano and Omori, 2009; Mano et al., 2012). In Arabidopsis, few genes have also been reported for aerenchyma formation under hypoxic conditions (Mühlenbock et al., 2007). In barley, a major QTL for aerenchyma formation has been reported on chromosome 4H. This QTL explained 44% of the phenotypic variance and it was identified from a double haploid population of barley produced from Yerong (waterlogging-tolerant) and Franklin (waterlogging-sensitive) varieties (Zhang et al., 2016). In another experiment, a major QTL on chromosome 4H has been identified for inducible aerenchyma formation using a double haploid population produced from barley and *Hordeum spontaneum* (Zhang et al., 2017). Interestingly, the positions of the QTL were the same, or close to the position of the QTL identified for this trait in the Yerong/Franklin population (Zhang et al., 2016; Zhang et al., 2017). Further investigations are needed in barley to identify the candidate genes linked to these QTLs for root aerenchyma formation.

2.4.3 QTL mapping for physio-morphological traits

Incorporation of flooding tolerance in crop plants genetically has been a major focus in many breeding programs. In these breeding programs several physiological and morphological traits have been targeted to identify QTLs in many plant species (Tanksley et al., 1989; Andersen and Lübberstedt, 2003; Fan et al., 2006; Cattivelli et al., 2008). For example, a major contributing gene for submergence was identified on chromosome 9 in rice (Xu and Mackill, 1996). Also, several minor QTLs have been recognized on four different chromosomes 6, 7, 11 and 12 (Nandi et al., 1997). Several QTLs were also detected in many studies by targeting the escape strategy in rice (Sripongpangkul et al., 2000; Toojinda et al., 2003; Nemoto et al., 2004; Tang et al., 2006; Hattori et al., 2008; Kawano et al., 2008).

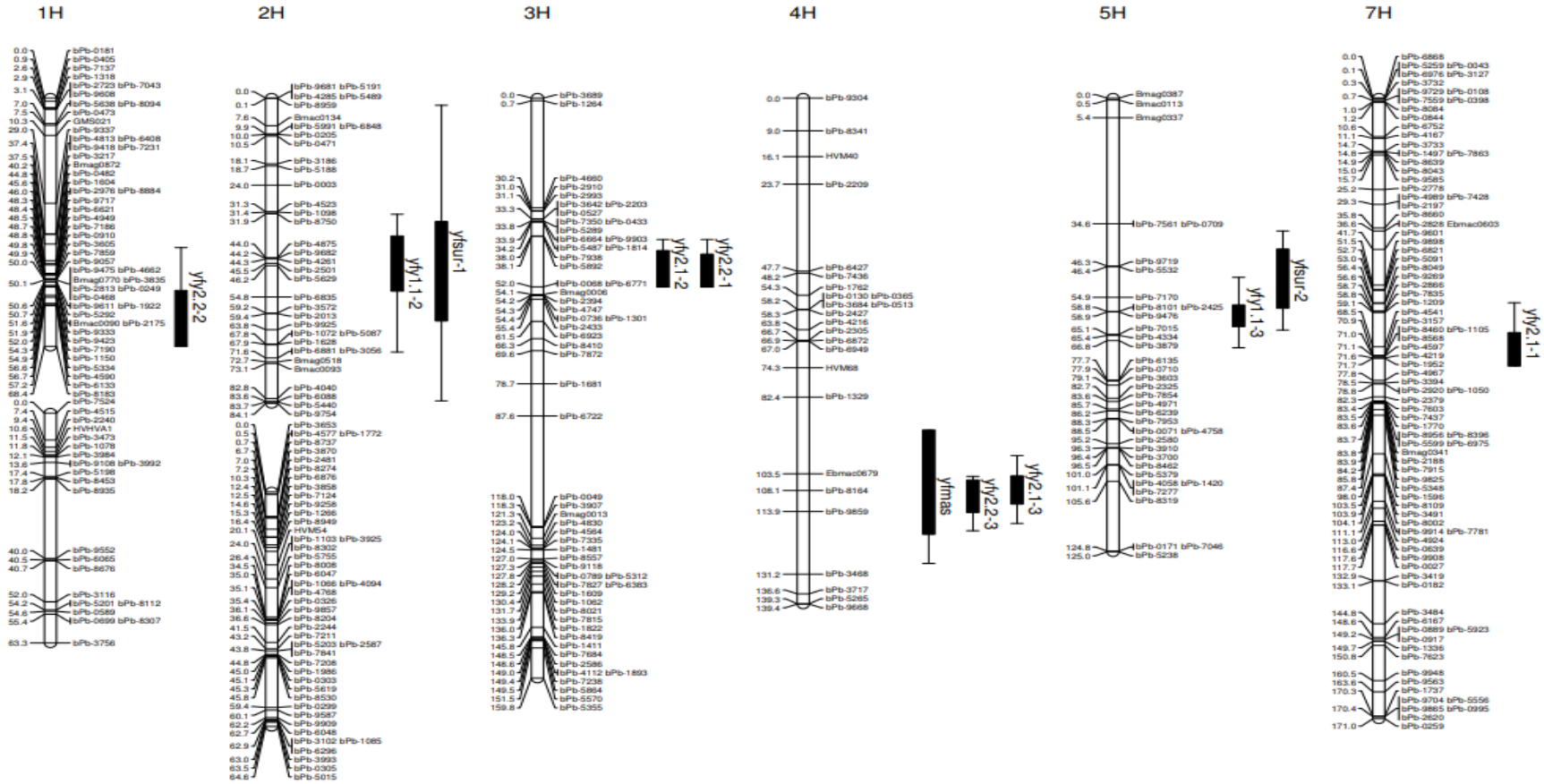

Fig. 2.5 Chromosomes showing the locations of QTL for different traits in barley. For leaf chlorosis QTL were identified on chromosomes 1H, 2H, 3H, 4H 5H and 7H. One QTL for plant biomass on chromosome 4H and two QTL for plant survival on chromosome 2H and 5H were also identified. This figure taken from Li et al., 2008, BMC Genomics, 9:401.

Many major and minor QTLs were also localised on eleven different chromosomes in rice for germination rate (Jiang et al., 2004; Angaji et al., 2010; Septiningsih et al., 2012). Two major QTLs were detected in maize for flooding tolerance on chromosome 1 by targeting leaf injury and dry matter production traits. Interestingly, the position of the QTL for leaf injury was the same as the QTL for dry matter production (Mano et al., 2006). The detection of these QTLs at the same position may suggest the potential use of this QTL to increase productivity by transferring flooding tolerance genes from a tolerant cultivar. Plant height, root dry weight, shoot dry weight and adventitious root formation were also targeted in maize and QTLs were detected on different chromosomes (Mano et al., 2005; Qiu et al., 2007). Identification of QTLs associated with waterlogging tolerance in soybean was also reported. In these reports germination rate, plant growth, gain yield, waterlogging score (achieved from ratio of leaf chlorosis and plant survival rate), and waterlogging tolerance index (combined plant height and total number of survived leaves after stress) traits were targeted to investigate QTLs (VanToai et al., 2001; Reyna et al., 2003; Cornelious et al., 2005; Wang et al., 2008; Fu et al., 2010).

Attempts have also been made to identify QTLs associated with waterlogging tolerance in wheat and barley by targeting morphological and physiological traits. QTLs have been successfully detected in wheat by targeting pre-harvest sprouting, root and shoot growth, and yield-related indices as measuring traits (Cornelious et al., 2005; Kulwal et al., 2005; Galindo, 2012; Wang et al., 2012). In barley, twenty different QTLs were identified for waterlogging tolerance. These QTLs were found in two barley double haploid (DH) populations TX9425 × Franklin and Yerong × Franklin (Li et al., 2008). Several yield components (grains per spike, spikes per plant, kernel weight and spike length) were targeted as indicators of waterlogging tolerance screening. Major QTLs related to these traits were located at linkage group 2H (Xue et al., 2010; Zhou, 2011; Xu et al., 2012; Zhou et al., 2012). Leaf chlorosis, plant height, plant biomass reduction, plant survival and waterlogging scores have also been targeted, and QTLs on different chromosomes in barley were identified (Li, 2007; Li et al., 2008; Xue et al., 2010; Zhou et al., 2012; Bertholdsson, 2013; Bertholdsson et al., 2015). In recent years, an overwhelming volume of information has been accumulated on the physiological, morphological, anatomical, molecular, biochemical, and metabolic responses to waterlogging stress in plants. Surprisingly, little progress has been made on achieving waterlogging stress tolerant varieties in plants including barley. However, this pyramiding goal can be achieved by targeting the traits directly related to mechanisms of waterlogging tolerance.

Chapter 3

The ability to regulate voltage-gated K^+-permeable channels in the mature root epidermis is essential for waterlogging tolerance in barley

3.1 Introduction

Waterlogging (WL) is a major environmental constraint limiting agricultural production worldwide and hampering 10% of the global land area (Setter and Waters, 2003). The estimated annual financial loss in agricultural production due to floods exceeds 60 billion euro (Mechler et al., 2010). Global climate changes are predicted to increase the rate and severity of flooding events in a large number of agricultural and urban areas of the world during this century (Arnell and Liv, 2001; Seneviratne et al., 2012; Hirabayashi et al., 2013). Under waterlogged conditions, the level of oxygen in the soil drops down rapidly from 230 nmol m^{-3} (well-drained soil) to 50 nmol m^{-3} (hypoxic) (Turner and Patrick, 1968) or may even result in a complete absence of oxygen (anoxia), due to high microbial activities (Ponnamperuma, 1984). Under these hypoxic and anoxic conditions, the resultant O_2 deficiency and accumulation of CO_2 in the root zone limit the root metabolism, aerobic respiration, and ATP synthesis, affecting the growth of shoots and roots (Gibbs and Greenway, 2003; Bailey‐Serres and Colmer, 2014). In addition, hypoxia also limits the availability of required energy to fuel the H^+-ATPase pumps and severely plunders the transportations of ions which ultimately affect the growth and yield (Bailey-Serres and Voesenek, 2008; Elzenga and van Veen, 2010).

Potassium (K^+) is the second most abundant mineral element in plant tissues. Although availability of K^+ differs from ~0.025mM to 5mM in different soils depending on the soil type and other environmental factors (Maathuis, 2009), cytosolic K^+ concentrations in plants are sustained at a level of ~150 mM (Kronzucker et al., 2008; Shabala et al., 2015) to enable the essential role of K^+ in activating and regulating of nearly 70 different metabolic enzymes in plants (Dreyer and Uozumi, 2011). Potassium also plays an important role as a determinant of the cell fate, with cytosolic K^+ acting as a trigger of the programmed cell death under a range of biotic and abiotic stress conditions (Shabala, 2009; Demidchik et al., 2010). The plant's ability to uptake K^+ is significantly reduced under oxygen‐deficient conditions (Kreuzwieser and Gessler, 2010; Zhao et al., 2012; Zeng et al., 2014), but detrimental effects of waterlogging

on plants may be ameliorated by the exogenous application of K^+ to soil or as a foliar spray (Ashraf et al., 2011; Wang et al., 2013).

Although the important role of maintaining intracellular K^+ homeostasis in plant adaptive responses to flooding stress has been repeatedly mentioned on many occasions (Pang et al., 2004; Colmer and Greenway, 2010; Mugnai et al., 2011; Shabala et al., 2015), some controversies were reported in previously published studies. While significant decline in the shoot K^+ content under waterlogged conditions was reported in many species (e.g. eucalypts - Close and Davidson, 2003; *Hordeum marinum* - Malik *et al.*, 2008; wheat - Trought and Drew, 1980; lucerne - Smethurst *et al.*, 2005; soybean - Board, 2008), results on roots are more controversial. The reported results range from substantial decline (e.g. lucerne - Smethurst *et al.*, 2005; wheat - Buwalda *et al.*, 1988; cucumber - Alcántara *et al.*, 1991; cherrybark oak - Pezeshki *et al.*, 1999) to no change or even increase (e.g. corn - Ashraf and Rehman, 1999; baldcypress - Pezeshki *et al.*, 1999; *Lotus tenuis* - Teakle *et al.*, 2010) in root K^+ content. In a study using two halophyte grasses (*Puccinellia ciliata* and *Thinopyrum ponticum*) differing in WL stress tolerance (Teakle et al., 2013), the WL-tolerant *P. ciliata* showed a significant uptake of K^+ under oxygen-deprived conditions, while more sensitive *T. poticum* showed a substantial K^+ leakage when measured by the microelectrode ion flux estimation (MIFE) system. The possible explanations for the above controversies may lay in the fact that both transport and regulation of K^+ homeostasis under oxygen-limited conditions should be put into the context of the root tissue- and genotypic- specificity. Also, even though the link between root's ability to retain K^+ and the WL stress tolerance has been reported (Pang et al., 2006; Zeng et al., 2014), the molecular mechanisms underlying this phenomenon remain unclear. K^+ transport across the plasma membrane (PM) is mediated by a very large number (75 in *Arabidopsis*) transport systems, and which of them plays a major role in hypoxia response remains to be elucidated.

The PM H^+-ATPases generate the proton motive force (Sondergaard et al., 2004) and thus are central to the maintenance of membrane potential and nutrient uptake by roots. Oxygen deficiency rapidly depolarizes plasma membrane potential of WL-sensitive species of higher plants (Buwalda et al., 1988; Teakle et al., 2013). As a result, the channel-mediated uptake of many essential cations (e.g. K^+, Mg^{2+}, and NH_4^+) is reduced or becomes thermodynamically not feasible. Many biotic and abiotic stresses induce K^+ leakage from plant tissues; in most cases, this K^+ efflux is mediated by depolarization-activated outwardly-rectifying K^+ (GORK)

channels (Demidchik et al., 2014; Shabala and Pottosin, 2014). The GORK channel displayed high expression in guard cells, root (epidermal cells, cortex and root hairs) and cells of the vascular tissue (Gambale and Uozumi, 2006; Ward et al., 2009). The expression and activity of GORK channels were both substantially affected under drought, salt and cold stress conditions (Becker et al., 2003), and these channels were named as a major pathway for stress-induced K^+ leakage in many plant species exposed to salt stress (Shabala and Cuin, 2008; Jayakannan et al., 2013). A pharmacological study suggested that the hypoxia-induced K^+ leakage may also be mediated by KOR channels, as K^+ efflux in barley roots was strongly inhibited (Pang et al., 2006) by the application of tetraethylammonium, a known blocker of the voltage-gated Shaker-type K^+ channel to which GORK belongs. In a recent study from our laboratory, we have shown (Wang et al., 2016) that the expression of *GORK* was down-regulated in hypoxic root cells of wild-type *Arabidopsis*. In addition, the mutant *gork1-1* lacking functional GORK channels showed significantly higher K^+ accumulation in both elongation and mature zones under hypoxia stress, compared with wild-type. After three days of hypoxia stress, *gork1-1* showed significantly smaller K^+ efflux than wild-type in the elongation zone and retained an influx in the mature zone; while, wild-type showed a K^+ efflux (Wang et al., 2016). The most likely explanation behind this tissue-specific K^+ efflux would be the differential regulation ability of GORK channels in the elongation and mature root zones. Can the same conclusion be extrapolated from *Arabidopsis* to cereals? Are all root cells equally sensitive to hypoxia, or some tissues may have a higher demand for oxygen? Can hypoxia-stress induced K^+ efflux be used as a physiological marker for WL stress tolerance? If yes – where should it be measured from?

The aim of this study was to address the above gaps in our knowledge. To achieve this, we used contrasting barley cultivars to investigate tissue- and genotype-specific effects of hypoxia on K^+ retention in roots. Also, the role of GORK channel in K^+ release was investigated by measuring hypoxia-induced changes in root MP and correlating it with the extent of K^+ efflux from the root. Our results showed that hypoxic conditions caused a significant loss of K^+ in a time- and genotype-specific manner, affecting cell viability and K^+ regulation, and led to the conclusion that the genotypic difference in waterlogging stress tolerance in barley is also contributed by the differential ability to regulate voltage-gated K^+-permeable channels in the mature root epidermis.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Six barley (*Hordeum vulgare* L.) cultivars contrasting in waterlogging (WL) tolerance were used in this study. Among these cultivars, CM72, TX9425 and Yerong are tolerant; Gairdner Franklin and Naso Nijo are sensitive to WL (Pang et al., 2004; Zhou et al., 2012). Seeds were acquired from China and the Australian Winter Cereal Collection Centre and reproduced in the field, using Tasmanian Institute of Agriculture (TIA) facilities in Launceston. Seeds were surface sterilized for 10 minutes with 10% commercial bleach (NaClO 42 g L^{-1}; Pental Products, Shepparton, Australia), thoroughly rinsed with tap water (for at least 30 min) and then grown in wet paper rolls with basic salt media (BSM) solution (0.5 mM KCl + 0.1 mM $CaCl_2$, pH 5.6) in the dark for 3 d, at room temperature (25 ± 1°C). Two treatments were used in electrophysiological experiments: (1) control (BSM, aerated); and (2) hypoxia (BSM solution made with 0.2% agar and bubbled with N_2 gas). For the treatment with agar, the stagnant solution was prepared by adding agar (Cat. No. LP0011, Oxoid, Hampshire, UK) to the BSM solution at a ratio of 0.2% (w/v) and boiled, then cooled down overnight at room temperature with magnetic stirring to prevent lump formation. The agar solutions were pre-bubbled with high purity N_2 (Cat. No. 032G, BOC Gases, Hobart, Australia) for at least 1 h before being used in the experiment. The concentration of oxygen in the hypoxic solution was zero when measured before applying to plants. This data is available from Zeng et al (2014 Plant Cell Environ) in Fig 8.

3.2.2 Glasshouse experiments

For glasshouse experiments, seeds of above six barley varieties were grown in 2 L pots filled with sandy loam soil. The soil was collected from University of Tasmania farm and mixed with essential macronutrients (in g/L: 0.51 NH_4NO_3, 1.35 NaH_2PO_4, 0.48 K_2SO_4, 0.31 $CaSO_4$, and 0.14 $MgCl_2$). During the germination stage, plants were watered with tap water at field capacity. Germinated plants were then thinned to eight uniform and healthy plants in every pot. Plants were grown under controlled glasshouse conditions (with a day-length of 14 h; light/dark temperatures of 25/15°C; and relative humidity of 65-75%) at the University of Tasmania (Hobart, Australia) in March-April 2014.

Treatments were imposed when plants were 10 d old. Two treatments were given: control (well aerated) and waterlogging (WL; submerged pots). For treatment with waterlogging, pots were placed into large containers (4 pots in each container). Waterlogging conditions were

imposed by using half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). The water level of waterlogged treatment was kept 15 mm above the soil surface. Control (well aerated) plants were irrigated on a daily basis with 150 mL of half-strength Hoagland's nutrient solution. Plants were subjected to treatments for four weeks. Leaf chlorophyll content was measured from the centre of the fully expanded leaves using SPAD meter (SPAD-502, MINOLTA, Japan). Before harvesting for biomass, the number (no.) of chlorotic, necrotic, and total leaves from each plant were counted. The relative amount of chlorotic and/or necrotic leaves was then calculated according to the equation: ratio of chlorotic (or necrotic) leaves = no. of chlorotic (/necrotic) leaves /no. of total leaves. Ten replicates were randomly taken for each treatment × variety combination**.** Fresh weight of shoots was recorded after harvesting, and the dry weight was measured after drying in a UniTherm Drier (Birmingham, England) for 2 d at 65 ˚C. Four replicates each consisting of four plants were used for each treatment.

3.2.3 Viability staining

For viability staining, surface-sterilized seeds of different barley cultivars were grown in aerated hydroponic BSM solution in the dark for 2 d at 25 ± 2 °C. On the next day, seedlings were transferred into 14/10 h light/dark regime for one day. The root area of plants was protected from direct light by using black containers. Two treatments - control and hypoxia (0.2% agar) - were imposed and lasted for 48 h. The cell viability was measured by using a fluorescein diacetate (FDA)-propidium iodide (PI) double staining method, principally as described by (Koyama et al., 1995). The settings of the camera for all experiments were kept constant during image acquisition. The green fluorescence signal intensity (a measure of the cell viability) was quantified by using ImageJ software version (1.48, Java, 64 bit).

3.2.4 Tissue elemental analysis

For tissue elemental analysis, plants were harvested after four weeks of treatment. Plant shoots were washed three times with distilled water and blotted to remove extra water. Plants were kept at 80°C for 48 h in an oven and then grounded into a powder. Plant samples (0.2 g) were digested with a mixture of 5 ml of HNO_3 + 1 ml of $HClO_4$ in infrared digestion furnace (PEIOU-SKD-20N, China). The resultant solutions were diluted to 25 ml using 2% HNO_3 and filtered with a (0.45 µm) filter paper. The concentration of K^+ in the filtrate was determined using inductively coupled plasma–atomic emission spectrometry (IRIS/AP optical emission spectrometer) following a standard procedure.

3.2.5 MIFE ion flux measurements

Net fluxes of K^+ and H^+ were measured from 60 ± 10 mm long roots by using a non-invasive ion flux measurement (the MIFE) technique (University of Tasmania, Hobart, Australia). The theory of MIFE measurements and other details of calibration and fabrication related to ion-selective microelectrode are given in our previous publications (Shabala et al., 2006; Shabala et al., 2010). In brief, borosilicate microelectrodes were filled with appropriate backfilling solution. Electrode tips were then filled with an appropriate Liquid Ion Exchanger (LIX) (Fluka Catalogue no. 60031 for K^+ and 95297 for H^+). After calibration in a set of pH and different K^+ standards, the electrodes were mounted on a 3D-micromanipulator. Electrodes were then positioned 40 µm to root surface and placed on the same plane 2-3 µm to each other. During the measurement of fluxes, microelectrodes were moving between two positions: 40 µm (close to root epidermis) and away (90 µm) in a 10-s cycle square-wave manner. The potential difference between these two points was recorded by the MIFE CHART software and converted into electrochemical potential difference by using the cylindrical diffusion geometry (Newman, 2001).

Prior to measurement a 3-d old seedling was taken from a paper roll and immobilized horizontally by the Plexiglass partitions 2 mm above from the surface of the chamber (see Fig. 3.1). The measuring chamber was filled with hypoxia solution while the coleoptile was kept above the surface of the solution. Seedlings for control were well aerated during the period of treatment; whereas hypoxia (0.2% agar) treated seedlings were kept in stagnant conditions for different timings, ranging from 2 to 48 h. The seedlings were then placed in a Faraday cage for MIFE measurements. Net ion fluxes were measured for 10 min; the steady-state fluxes were achieved by averaging the values of the last 5 min. For each treatment, net ion fluxes were measured from intact roots of at least 6-8 individual seedlings.

In some experiments, excised roots were used to eliminate the possibility of oxygen supply through coleoptiles and to restrict internal oxygen movement. For this purpose, a 3-day old seedling was taken from a paper roll and the coleoptile was removed gently with a scalpel blade. The excised roots were placed in a Faraday cage for MIFE measurements after giving different treatments as described in the previous paragraph. For each treatment, net ion fluxes were measured from 6-8 individual roots with excised coleoptiles.

3.2.6 Membrane potential measurements

Membrane potential (MP) was measured from epidermal cells of intact barley roots. The MP measurements were performed as described previously (Cuin and Shabala, 2005; Bose et al., 2010). Briefly, conventional microelectrodes (Harvard Apparatus) were filled with 1 M KCl and connected to MIFE electrometer via Ag/AgCl half-cell. During MP measurement, the microelectrode with a tip diameter of 0.5 µm was impaled into the epidermal cells of mature root zone with a manually functioned 3D-micromanipulator (MHW-4, Narishige, Tokyo, Japan). MP values were recorded by the MIFE CHART software for at least two minutes after stabilization (Newman et al., 2001). For MP measurements, the barley seedlings were mounted in the vertical chambers and treated with a hypoxia solution as described for MIFE experiments. For each treatment, MP values were measured from the roots of 5-6 individual seedlings. At least four different cells were measured for each seedling. MP measurements were made either before (time zero) or after 48 h of treatment.

3.2.7 Pharmacology

Roots pre-treated with hypoxia stress (0.2% agar) for 24 h and control roots were used for pharmacological experiments. Net ion fluxes were measured for five minutes both in control and hypoxia treated roots, then 0.5 mM orthovanadate (a potent inhibitor of H^+-ATPase pump) was applied and steady-state fluxes were recorded for another 10 minutes. For each treatment, net ion fluxes were measured from roots of at least 6-8 individual seedlings.

3.2.8 Quantitative real-time PCR

RNA from the root apices (4-5 mm long) from both mature and elongation zones of barley was isolated using the ISOLATE II RNA Plant Kit (Bioline) and purified by ISOLATE II micro clean-up kit (Bioline) according to the manufacturers' protocols. 8000 ng total RNA was reverse transcribed using Sensi FAST cDNA Synthesis Kit (Bioline) in a total volume of 20 µl. RNA concentration was monitored with the Nano Drop 8000 UV-Vis Spectrophotometer (Thermo Scientific). Quantitative real-time PCRs (qRT-PCRs) were performed in a Roto-Gene 3000A (Corbett Research) with the Quanti Nova SYBR Green PCR Kit (Qiagen) in a 20 µl volume containing 2 µl of 5-fold diluted cDNA and 0.7 nM of primer mix (1:1 mix of forward and reverse primers). After a hot start (5 min at 95°C), a two-step PCR program was applied: 45 times for 10 s at 95°C, and 30 s at 60°C, followed by a dissociation curve by increasing the temperature every 1°C from 55 to 99°C (with a 5-s hold at each temperature). Primers (Gene Works) are listed in Table 3.1. All quantifications were normalized to 10 000 molecules of the

housekeeping gene *HvGAPDH* and each transcript was quantified using individual standards. For each gene, the quantification in the mature and elongation zones of control conditions was also normalized to 1 (corresponding to 100%). Three to five technical and biological replicates were performed for each experiment and treatment.

Table 3.1 Primers used in the gene expression analysis

Gene name	Forward primer	Reverse primer
HvGAPDH	5'-GTGAGGCTGGTGCTGATTACG-3'	5'-TGGTGCAGCTAGCATTTGAGAC-3'
HvPMHA	5'-GCTGGTGTTATCTGGCTCTTC-3'	5'-CTCTTCTCTTGGCTT GCTCAG-3'
HvGORK	5'-CCACACGAGGCGAAGAAG-3'	5'-GAGGAATCCACAGCATCACC-3'
HVP1	5'-GAAGACTGTGCATAGCTGGC-3'	5'-ACATTGGTAGCAGCTCCAGT-3'
HVP10	5'-AGATGACCCAAGGAACCCAG-3'	5'-GCAAAGAGTGTGGTGAGCAA-3'

3.2.9 Statistical analysis

Statistical analysis was performed by a statistical package IBM SPSS Statistics 20 (IBM, New York, NY, USA). All data in the table and figures are given as means ± SE. The significant difference between mean was evaluated by Duncan's multiple range test.

3.3 Results

3.3.1 Genotypic variation in plant growth under waterlogging

A glasshouse experiment was undertaken to assess the genotypic variation in a range of barley genotypes under waterlogging stress. Four weeks of waterlogging stress affected the growth of all cultivars but to differing extents. The most severe effect of WL on plant growth was observed in cultivars Gairdner, Franklin and Naso Nijo (Fig. 3.2). These varieties were therefore termed as WL-sensitive, in contrast to the other three termed as WL-tolerant. When these cultivars were grown in sandy loam soil for 4 weeks, the average shoot FW of tolerant and sensitive cultivars were reduced by 42% and 68% relative to the control, respectively; for shoot DW these values were 32% and 61% (Fig. 3.2A, B). The chlorophyll content in the tolerant and sensitive group of cultivars was reduced by 9% and 41% as compared to respective controls (Fig. 3.2C). The effect of WL treatment on chlorophyll content (SPAD value) was not significant ($P<0.05$) in tolerant cultivars TX9425 and Yerong (Fig. 3.2C). Results showed that

WL treatment induced the chlorosis and necrosis of leaves, and the tolerant cultivars performed much better as compared to sensitive ones (Fig. 3.2D). There were no chlorotic and necrotic signs on leaves of waterlogging tolerant cultivars, while in sensitive cultivars 21% chlorotic and 26% necrotic leaves were observed on an average under waterlogging treatment (Fig. 3.2D).

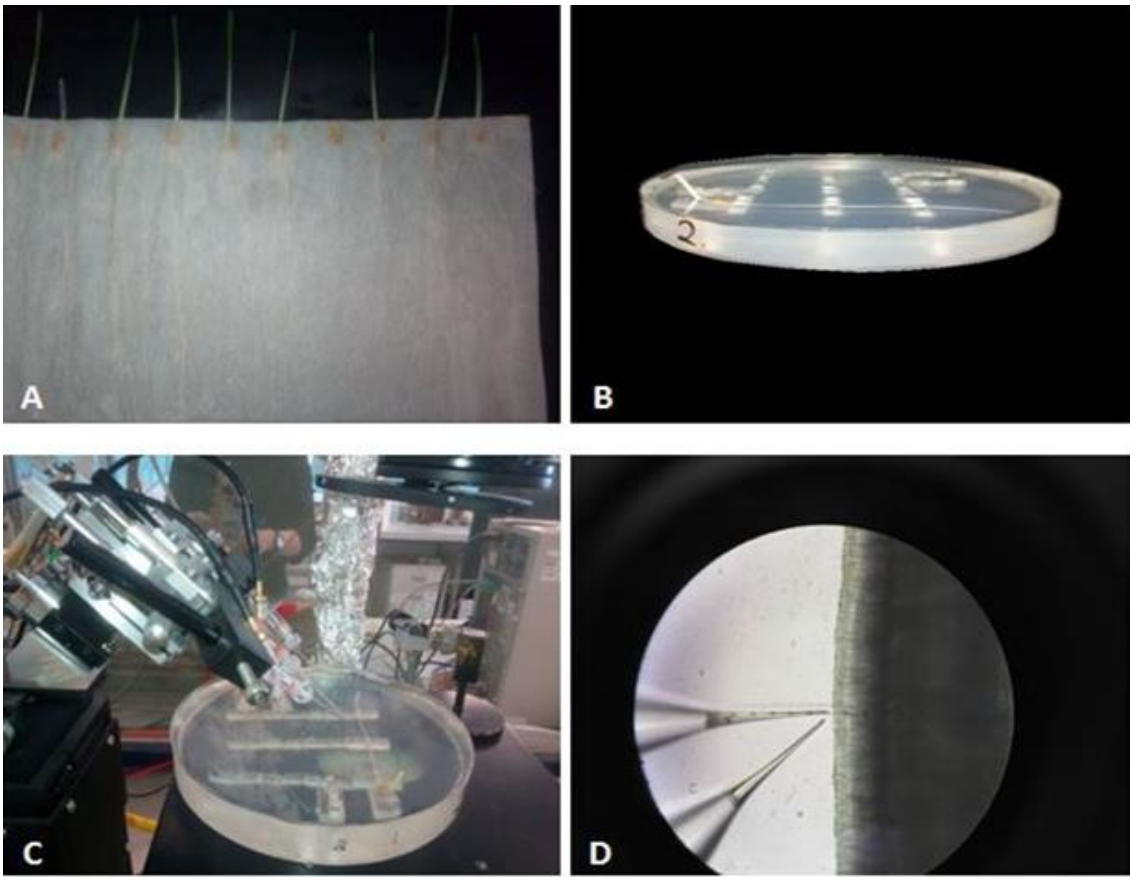

Fig. 3.1 Different steps of experimental procedures. (A) Plants are grown in paper rolls. (B) Plants are fixed in a chamber and treated with 0.2% agar for different timings. (C) Plant seedling is transferred to Faraday cage. (D) Ion selective electrodes are positioned for the measurement from the root epidermis.

3.3.2 Hypoxia-grown barley cultivars differed in cell viability and whole-plant K^+ content

We next used the viability staining technique to comprehend the tissue-specific effects of hypoxia on cell metabolism (Fig. 3.3). A significant ($P<0.05$) loss in the viability was found in both elongation and mature zone after hypoxia (Fig. 3.3B). The apical cells were more severely damaged as compared to mature zone (as indicated by the deep red colour in Fig. 3.3A). However, a very strong heterogeneity of the fluorescent signal from the apex (Fig. 3.3A) has questioned the suitability of this zone as a physiological marker in screening programs. In

this context, the signal from the mature zone is more uniform and thus more suitable for the screening purpose. Thus, we have used this region for comparing effects of hypoxia on root cells viability amongst six contrasting barley cultivars (Fig. 3.4A). There was no sign of any viability loss in tolerant cultivars in the mature zone but clear signs of the viability loss were observed in sensitive cultivars when compared with appropriate controls (Fig. 3.4A).

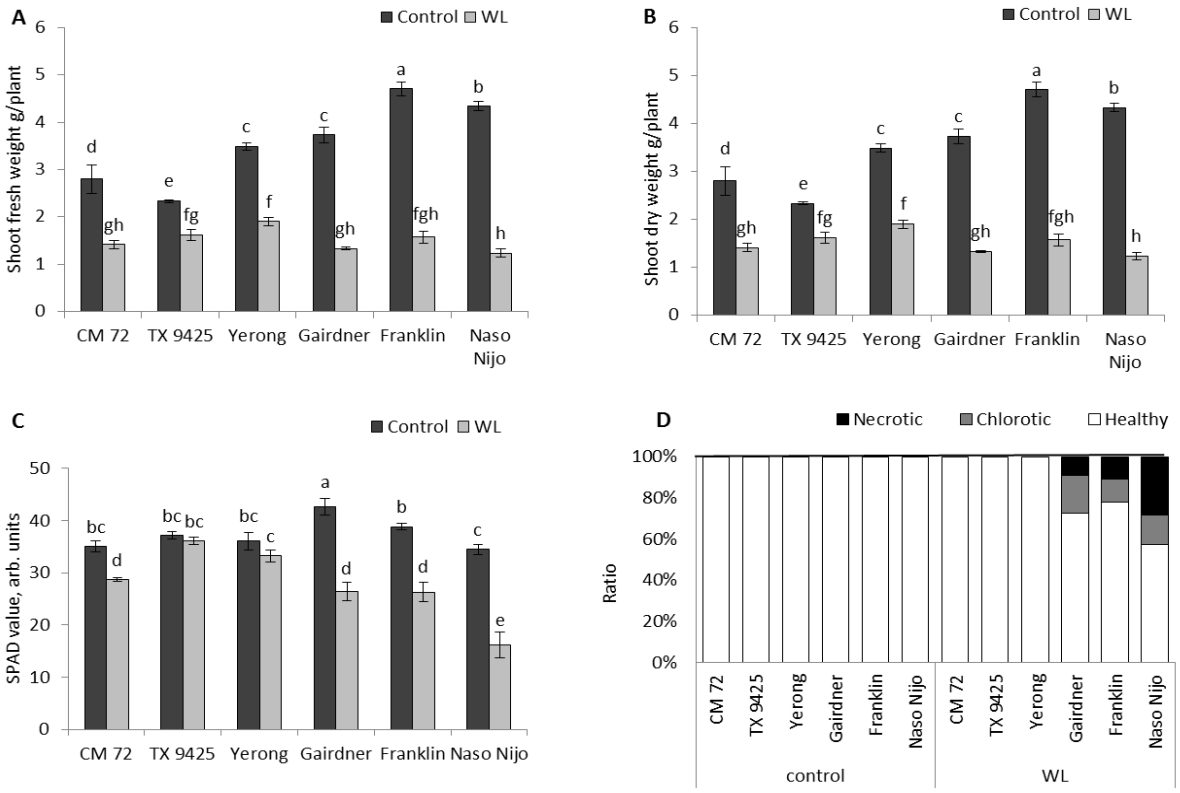

Fig. 3.2 Effects of waterlogging on agronomical and physiological characteristics of six barley cultivars. (A) Shoot fresh weight; (B) Shoot dry weight; (C) Chlorophyll content (SPAD values); (D) The ratio of chlorotic and necrotic leaves. Dark bars, control; light bars, waterlogging. Values are means ± SE (n = 4). Different lowercase letters indicate the significant difference at $P \leqslant 0.05$ according to Duncan's multiple range tests.

The above visual observations were then quantified revealing statistically significant (at $P<0.05$) difference between two groups (Fig. 3.4B, C). In sensitive cultivars, the loss of viability was almost 1.5-2 folds greater than tolerant cultivars (Fig. 3.4C). The loss of cell viability in hypoxia-treated roots mirrored the changes imposed by WL stress in shoot K^+ content of glasshouse-grown barley cultivars (Fig. 3.4D). Here, sensitive cultivars lost 1.5 to 2-fold more K^+ in the shoot, as compared to tolerant cultivars.

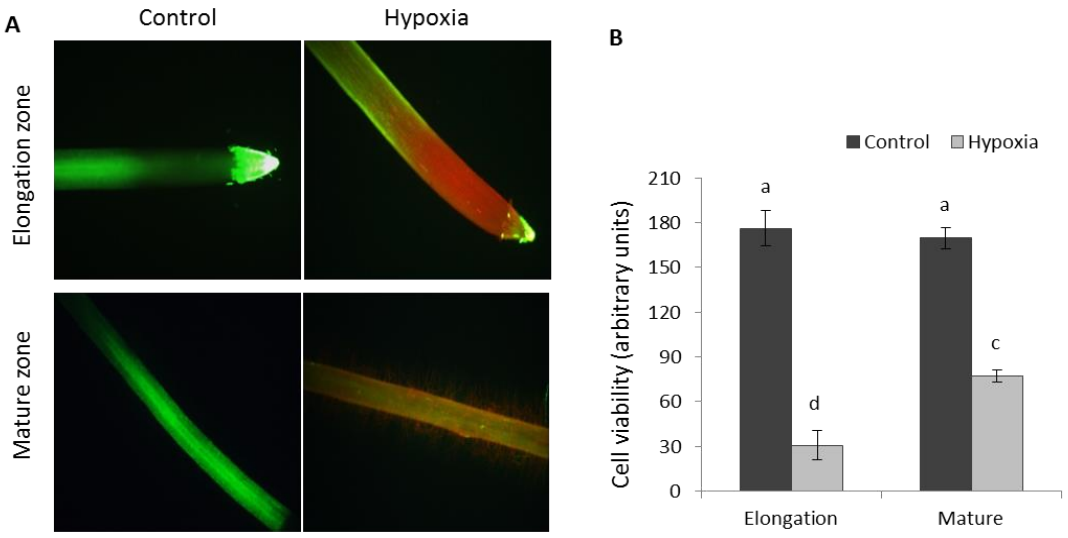

Fig. 3.3 Effects of hypoxia (N_2-bubbled 0.2% agar) on root viability of a waterlogging sensitive barley cultivar (Gairdner). (A) Barley roots were stained with fluorescein diacetate-propidium iodide (FDA-PI). Control and 48 h hypoxia treated barley roots stained with FDA (5 µg mL^{-1} for 5 min) and PI (3 µg mL^{-1} for 10 min) for fluorescence imaging. Viable cells displayed green fluorescence due to FDA, and non-viable cells displayed red fluorescence due to PI. One (of six) typical image is shown for each zone (elongation zone, ~3 mm from root tip; mature zone, ~5-10 mm from the shoot base). (B) Quantification of the cell viability from mature and elongation zones of barley roots. The intensity of the green fluorescence signal (a measure of cell viability) was quantified by Image J software. Data are mean ± SE (*n* = 6-8 individual plants). Scale bar = 0.5 mm. Different lowercase letters indicate the significant difference at $P \leq 0.05$ according to Duncan's multiple range tests.

3.3.3 Long-term hypoxic treatment is more detrimental to root ion fluxes

Net K^+ and H^+ fluxes were measured from intact barley roots to examine effects of hypoxia on ion flux profile along the root axis. Different treatment durations were used, ranging between 2 and 48 h. A significant uptake of K^+ in the mature zone and a strong efflux in the elongation zone were observed under control conditions (Fig. 3.5A). Hypoxic treatment for 2 h increased net K^+ efflux in the elongation zone; no significant change in net K^+ flux was observed in the mature zone (Fig. 3.5A) at this time point. As hypoxia progressed, both K^+ uptake (in the mature zone) and leakage (in the apex) were gradually reduced (24 h and 48 h hypoxic treatments). In the mature zone, no significant change was found for net H^+ efflux while a considerable reduction of H^+ influx was observed in the elongation zone of intact roots after two hours of hypoxia treatment (Fig. 3.5A). Steady-state H^+ flux gradually reduced in both zones as the hypoxia treatment progressed.

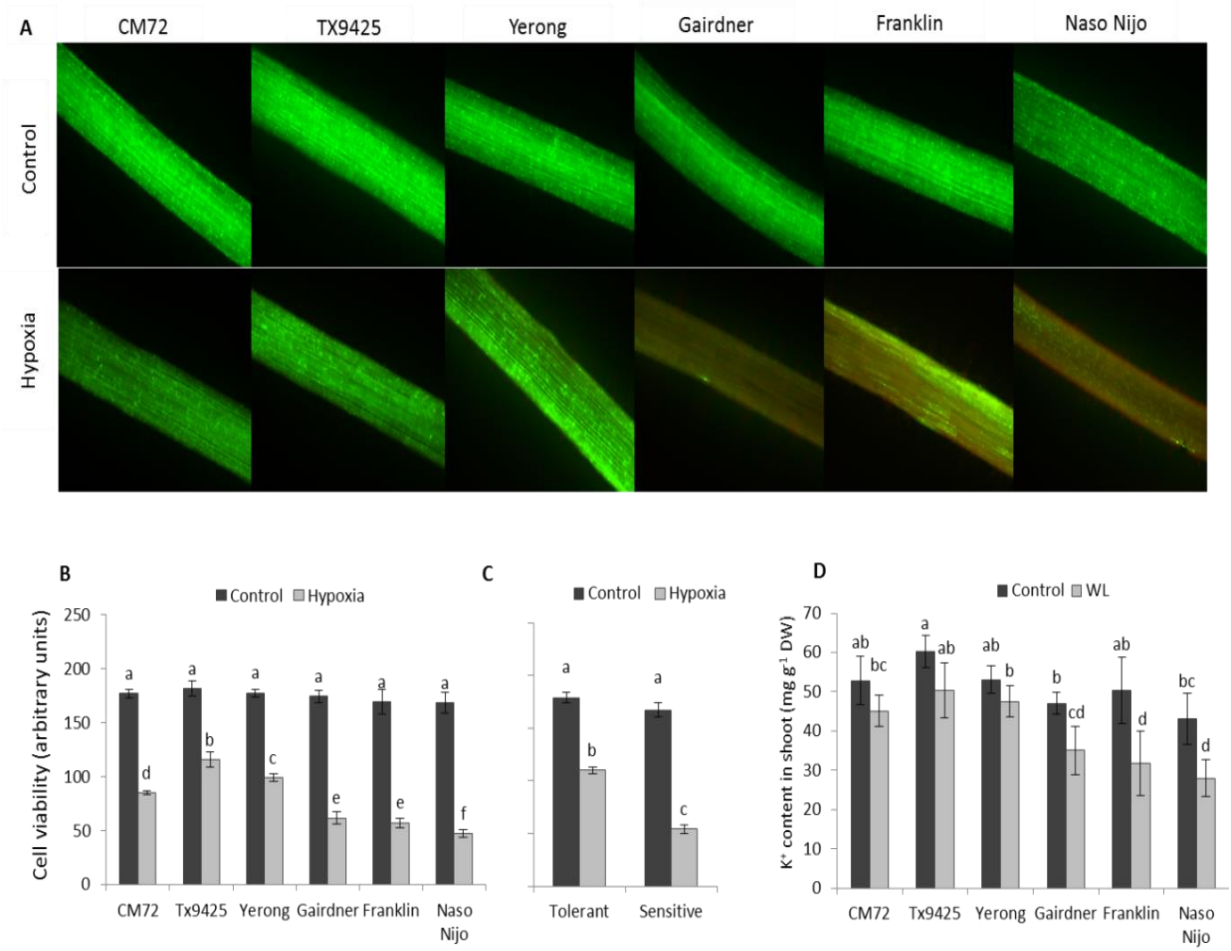

Fig. 3.4 (A) Genotypic variation in effects of hypoxia (N_2-bubbled 0.2% agar) on root viability of six barley cultivars different in waterlogging stress tolerance. Barley roots were stained with fluorescein diacetate-propidium iodide (FDA-PI). Viable cells displayed green fluorescence due to FDA, and non-viable cells displayed red fluorescence due to PI. One (of six) typical image is shown for each cultivar in the mature zone, ~5-10 mm from shoot base. The intensity of green fluorescence signal (a measure of cell viability) was quantified by Image J software. Scale bar = 0.5 mm. (B) Quantification of the cell viability from the mature root (5-10 mm shoot base). (C) Mean pooled cell viability quantification values for 3 tolerant and 3 sensitive varieties measured as above (D) Effects of four weeks waterlogging stress on K^+ content in shoots of six contrasting barley cultivars grown in sandy loam soil (mg g^{-1} DW). Data are mean ± SE (n = 6-8 individual plants).

A series of experiments were further conducted to verify the genotypic specificity of the detrimental effects of hypoxia on H^+ and K^+ fluxes (Fig. 3.5 B-D) to see if hypoxia-induced ion fluxes could be used as a proxy for waterlogging stress tolerance. The above six barley cultivars were used to compare ion fluxes under hypoxic conditions (48 h treatment). Measurements were conducted in the mature zone, 5 mm from the shoot base (Fig. 3.4). Hypoxia reduced the rate of H^+ pumping and resulted in a significant decline in K^+ uptake after

48 h of hypoxia (Fig. 3.5B, D). Tolerant cultivars showed significantly better abilities for H^+ pumping and showed less decrease in K^+ uptake than the sensitive ones under both control and hypoxic condition (Fig. 3.5C, E), with tolerant cultivars showing 30% less K^+ leakage (Fig. 3.5C) and 40% more H^+ pumping (Fig. 3.5E) as compared to sensitive cultivars. The observed decrease in shoot K^+ content showed strong ($R^2 = 0.85$) positive correlation with net K^+ efflux from stressed roots measured in MIFE experiments (Fig. 3.6).

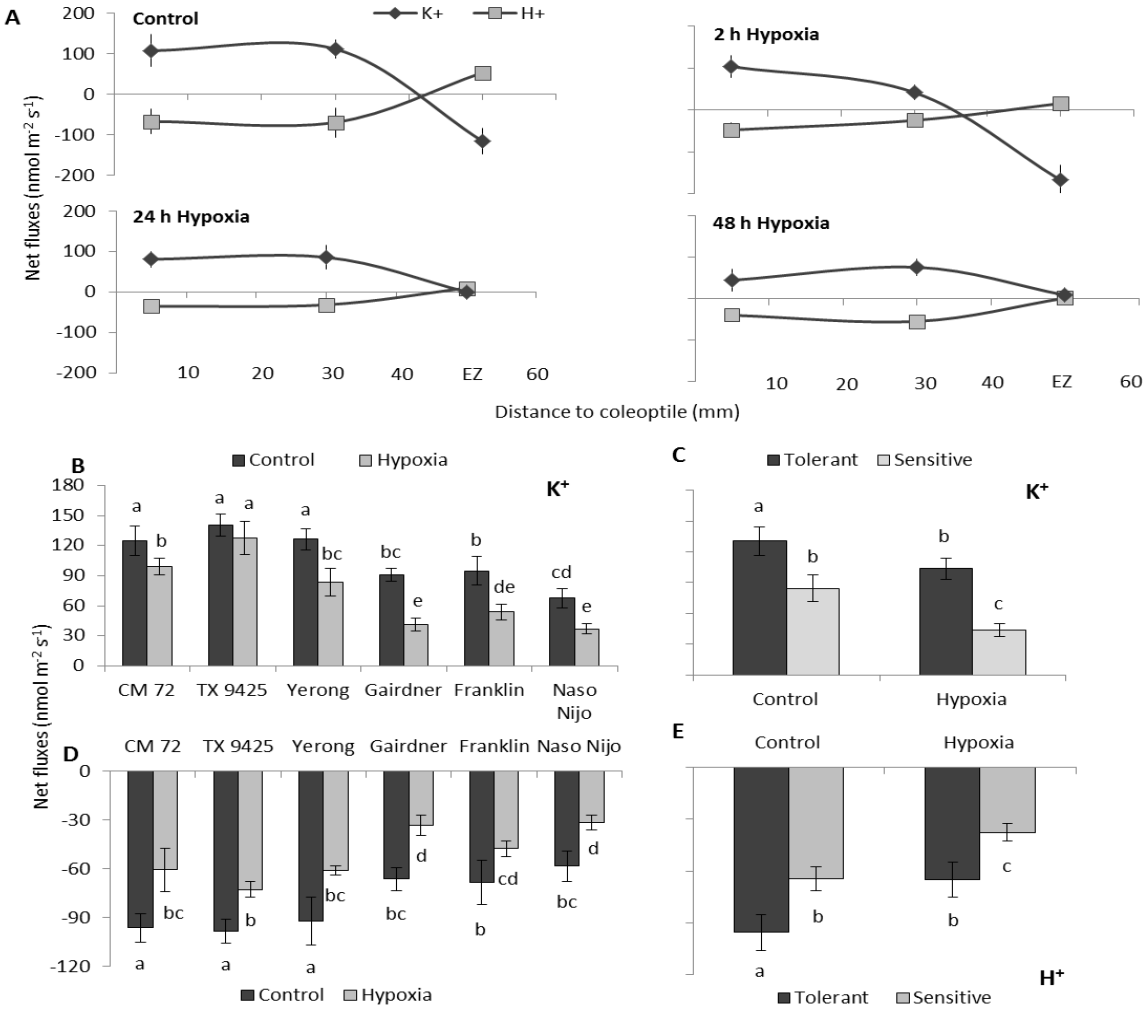

Fig. 3.5 Effects of hypoxia (N_2-bubbled 0.2% agar) on net K^+ and H^+ fluxes measured from barley root epidermis of intact seedlings. (A) Mean fluxes were measured from the intact roots of a waterlogging sensitive barley cultivar (Gairdner) in control and after 2, 24 and 48 hours of hypoxia treatment from different zones along the root axis. EZ stays for elongation zone in the figure. (B) Net K^+ flux measured after 48 hours of hypoxia treatment in the mature root zone (5 mm from root base) of six barley cultivars contrasting in waterlogging tolerance. (C) Mean pooled K^+ values for 3 tolerant and 3 sensitive varieties measured as above. (D) Net H^+ flux measured after 48 hours of hypoxia treatment in the mature root zone (5 mm from root base) of six barley cultivars contrasting in waterlogging tolerance. (E) Mean pooled H^+ values for 3 tolerant and 3 sensitive varieties measured as above. Data are mean ± SE (*n* = 6-8 individual plants).

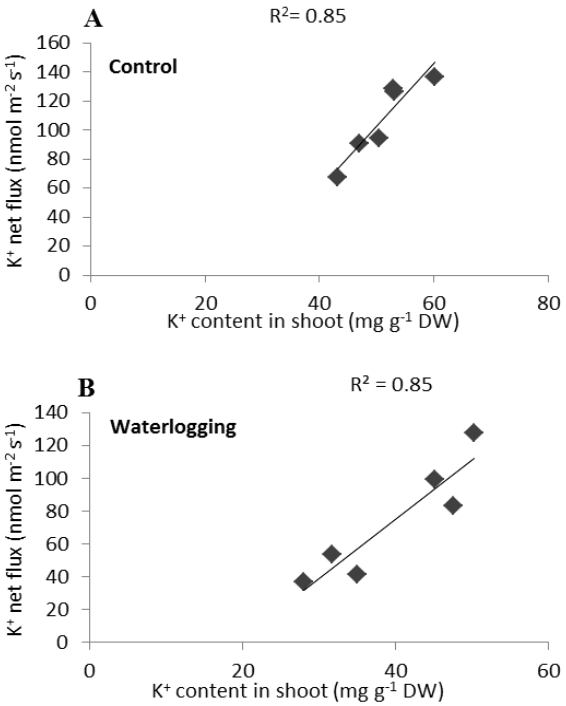

Fig. 3.6 Correlation between K^+ content in the shoot (mg g^{-1} DW) under waterlogging stress and net K^+ flux (nmol $m^{-2}s^{-1}$) in the mature root zone under hypoxia stress. Each point represents the mean value for one of six cultivars mentioned in the text.

3.3.4 Disruption of internal O_2 transport reduces ion fluxes under hypoxic conditions

In above experiments, measurements were conducted on intact seedlings, with their coleoptiles being in the air and thus operating as a "snorkel" supplying oxygen to root tissues even in the absence of oxygen in the growth media (Zeng et al., 2014). Thus, as a next step, we have investigated the effect of hypoxia on ion fluxes in plants where internal oxygen supply was prevented by excising coleoptiles. Net ion fluxes were measured from 10 different positions along the root axis (Fig. 3.7). A strong net H^+ efflux and K^+ uptake was measured from the mature zone of excised roots under control conditions (Fig. 3.7A). In contrast to the mature zone, the elongation zone showed an influx of H^+ and an efflux of K^+ (positions PE= pre-elongation, DE= distal elongation and M= mature zone in Fig. 3.7A). The onset of hypoxia resulted in a reduced K^+ uptake in the mature zone and further increased K^+ leak from the root apex after 2 h of hypoxia treatment (Fig. 3.7B). This K^+ loss in the apex was found to be transient and stopped after 24 h (Fig 3.7A). In the mature zone net K^+ uptake was reduced to zero or even turned into net efflux after 48 h (Fig. 7A). The steady-state H^+ flux also reduced considerably after 2 h of hypoxia treatment in both zones as compared to control (Fig. 3.5A). A significant reduction in H^+ flux was measured as hypoxia progressed in both mature and elongation root zones after long-term hypoxic treatments (24-h, 48-h). To understand the nature of transport systems mediating the observed effects of hypoxia on K^+ fluxes in barley roots, a correlation analysis between K^+ and H^+ fluxes was conducted. A strong correlation between

H^+ and K^+ fluxes existed at each time point (Fig. 3.7B) suggesting that the measured K^+ fluxes were mediated by some voltage-gated transport system.

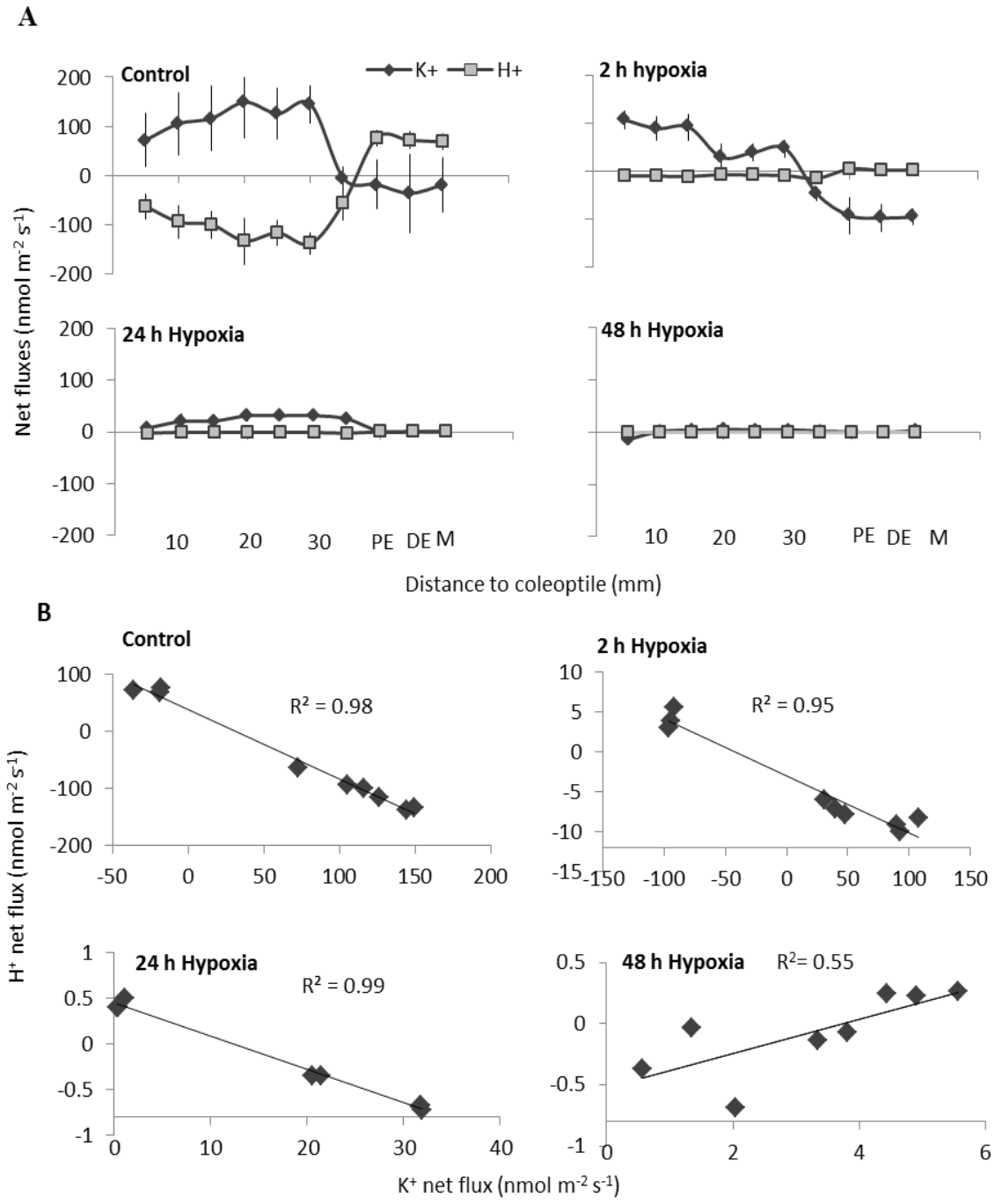

Fig. 3.7 Net K^+ and H^+ fluxes response to hypoxia (N_2-bubbled 0.2% agar) measured from excised roots of a waterlogging sensitive cultivar (Gairdner). (A) Mean fluxes were measured along barley root axis in control and after 2, 24 and 48 hours of hypoxia treatment from different zones. P = pre-elongation, D = distal-elongation and M = meristem. (B) Correlation between net K^+ and H^+ fluxes in control and hypoxia-treated roots measured at different time points. Data are mean ± SE (*n* = 6-8 individual plants).

Given the fact that preventing internal oxygen transportation by excising coleoptiles results in a stronger effect on ion profiles along the root axis, we next looked at the genotypic variation in this trait amongst barley genotypes, comparing effects of 48 h hypoxia treatment on K^+ and H^+ fluxes from mature zone in six contrasting cultivars (Fig. 3.8). The detrimental effect of hypoxia was much stronger in sensitive cultivars as compared to tolerant ones (Fig. 3.8B, D). The observed difference was much more pronounced in plants with excised roots (Fig 3.8) compared with intact plants (Fig 3.5B), suggesting that the former are more suitable to be used in screening barley germplasm for waterlogging stress tolerance by the MIFE technique.

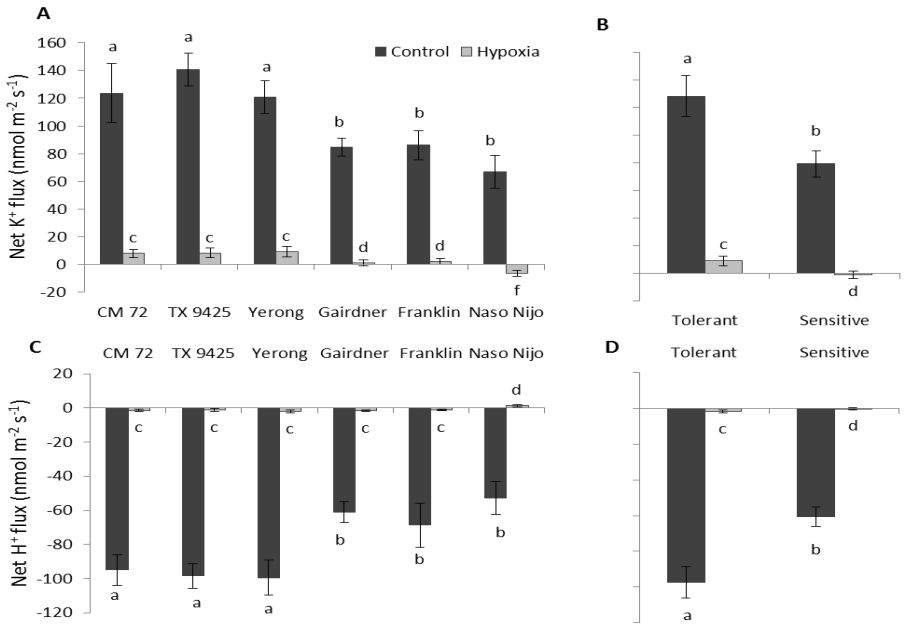

Fig. 3.8 Effects of hypoxia (N_2-bubbled 0.2% agar) on net K^+ and H^+ fluxes measured from excised roots of six barley cultivars contrasting in waterlogging stress tolerance. (A) – steady-state mean K^+ fluxes (averaged over five minutes) measured after 48 hours from mature root zone (5 mm from shoot base) of six contrasting cultivars from plants with excised coleoptiles. (B) – Mean pooled K^+ values for 3 tolerant and 3 sensitive varieties measured as above. (C) steady-state mean H^+ fluxes (averaged over five minutes) measured after 48 hours from mature root zone (5 mm from shoot base) of six contrasting cultivars from plants with excised coleoptiles. (D) Mean pooled H^+ values for 3 tolerant and 3 sensitive varieties measured as above. Data are mean ± SE (n = 6-8 individual plants). Different lowercase letters indicate the significant difference at $P \leq 0.05$ according to Duncan's multiple range tests.

3.3.5 WL tolerant cultivars maintain more negative membrane potential

K^+ leakage under stress conditions is largely mediated by the Shaker-like K^+ channels (GORK in *Arabidopsis*; Pottosin and Shabala, 2014). Most Shaker-like channels are strongly voltage-gated in nature. The GORK channel is not an exception and is classified as depolarization-

activated outward-rectifying K^+ channel (reviewed in Anschütz *et al.*, 2014). Accordingly, we have measured MP of epidermal root cells to check if the extent of hypoxia-induced K^+ leak is correlated with stress-induced changes in a membrane potential. Our data showed that tolerant cultivars maintained more negative MP values as compared to sensitive cultivars, both under control and hypoxic conditions (Fig. 3.9A). When pooled, MP values in the sensitive group were almost 10% less negative than in the tolerant group (Fig. 3.9B). MP depolarization was found to be consistent with the lower steady state H^+ flux in the hypoxic conditions (Figs 3.5D, E). This reduction in MP values under both conditions points to the involvement of the voltage-gated outward-rectifying K^+ channels as the major path for the observed stress-induced K^+ leak. This conclusion is further supported by pharmacological experiments using vanadate, a known blocker of the H^+-ATPase. In the mature zone, a significant reduction in H^+ efflux was measured in control roots while the efflux changed into an influx after vanadate application in the hypoxia-treated roots (Fig. 3.9D). Much higher H^+ influx was measured in the elongation zone when vanadate was applied to both control and hypoxia treated barley roots (Fig. 3.9C).

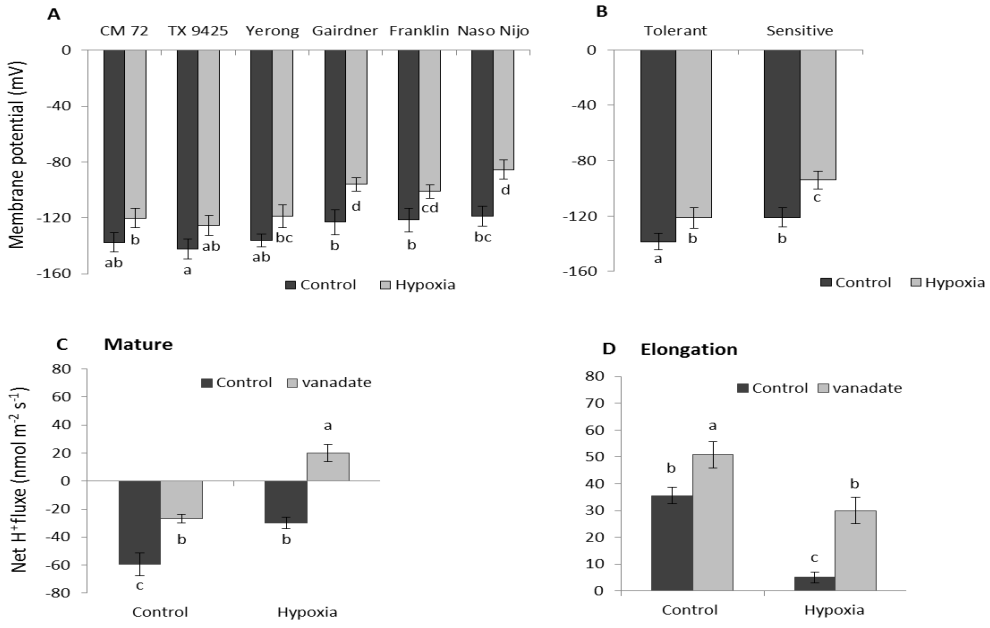

Fig. 3.9 (A) Effects of hypoxia (N_2-bubbled 0.2% agar) treatment after 48 hours on membrane potential of six contrasting barley cultivars measured from mature root zone (5 mm from shoot base). Data are mean ± SE (*n* = 18 to 30 measurements from at least five individual plants). (B) Mean pooled membrane potential values for 3 tolerant and 3 sensitive varieties measured as above (C, D) Effect of vanadate (0.5 mM) on steady-state H^+ fluxes measured from the mature and elongation regions of barley waterlogging sensitive cultivar (Gairdner) roots. Data are mean ± SE (*n* = 6 individual plants). Different lowercase letters indicate the significant difference at $P \leqslant 0.05$ according to Duncan's multiple range tests.

3.3.6 Hypoxic-induced changes in relative expression of *GORK* and H^+-pump genes

We next compared the relative expression pattern of GORK genes and genes conferring plasma membrane and tonoplast H^+-ATPase (HA) and H^+-pyrophosphatase (VP) pumps, in two root zones. The relative transcript level of the H^+-ATPase (*HvPMHA*) under hypoxia condition showed a comparable pattern in the mature and elongation zone (Fig. 3.10A), reducing the expression by 53% and 42%, respectively. This reduction in the gene expression matches nicely the reported 40-60% reduction in net H^+ flux measured in MIFE measurements (Fig. 3.5A, D). By applying hypoxia, the relative expression of the K^+ outward rectifier *HvGORK* decreased about 80% in the mature zone (Fig. 3.10B). On the contrary, the *HvGORK* transcript level in the elongation zone showed a pronounced increase under hypoxia treatment, which resulted in a 10-fold higher *HvGORK* expression relative to the non-treated elongation zone. For both vacuolar H^+-PPases (*HVPs*) the relative expression in the mature zone decreased by over 60% under hypoxia conditions (Fig. 3.10C, D), whereas in the elongation zone the hypoxia treatment resulted in a minor change of the *HVP1* expression. Whereas, in the elongation zone, the relative expression of *HVP10* was significantly higher (2.5 fold) under hypoxia treatment compared with the control condition.

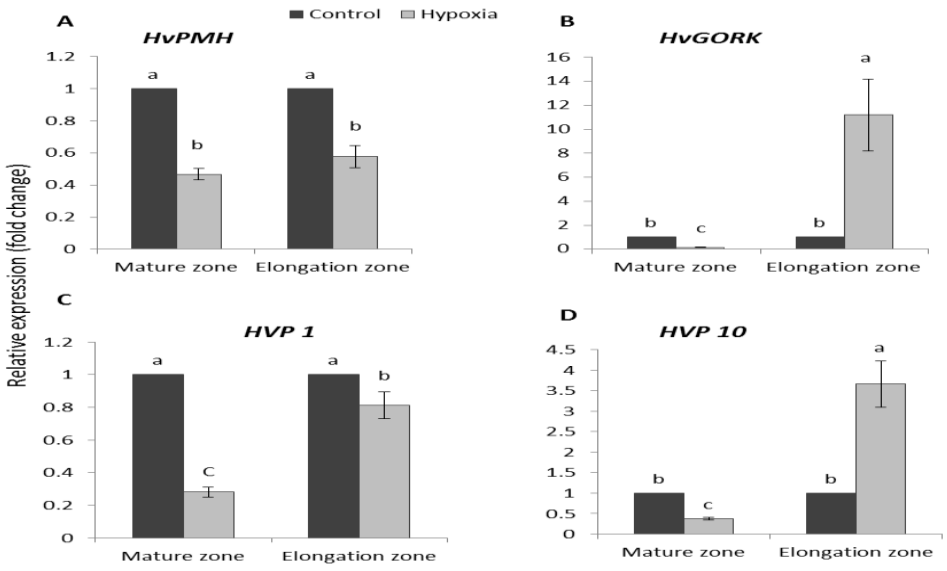

Fig. 3.10 Effects of 48 h hypoxia (N_2-bubbled 0.2% agar) treatment on relative expression of (A) H^+-ATPase (*HvPMHA*), (B) KOR (*HvGORK*), (C) H^+-HPPase (*HVP 1*) and (D) (*HVP 10*) in root tissues of waterlogging sensitive barley cultivar (Gairdner). Data are mean ± SE (*n* = 3-5 biological and technical replicates). Root apices (4-5 mm) long from both elongation (elongation zone, ~3 mm from root tip) and mature zone (mature zone, ~5-10 mm from the shoot base) were taken for relative expression analysis. Different lowercase letters indicate the significant difference at $P \leqslant 0.05$ according to Duncan's multiple range tests.

3.4 Discussion

3.4.1 K^+ retention confers cell viability and contributes to genotypic difference in WL tolerance in barley

Potassium is the most abundant inorganic cation in plant cells that play a crucial role in numerous physiological processes including turgor maintenance, regulation of enzymatic activity, maintenance of membrane polarization, control of sugar and ion loading, and energy conservation processes (Dreyer and Uozumi, 2011; Shabala et al., 2014). Potassium also plays an important role in plant adaptive responses to a hostile environment. The ability of plant tissue to retain K^+ was positively correlated with salinity stress tolerance in a broad range of plant species including barley (Chen et al., 2005), tomato (Heuer, 2003), wheat (Colmer et al., 2006; Cuin et al., 2008), lucerne (Smethurst et al., 2008), rice (Ismail et al., 2007), and poplar (Sun et al., 2009). Early studies in our laboratory have also indicated that K^+ retention ability in barley roots may be linked with waterlogging stress tolerance (Zeng et al., 2014). This suggestion is now fully validated by screening more barley cultivars differing in WL tolerance. We showed that the overall plant performance under waterlogged conditions (Fig. 3.2) correlated with whole plant K^+ tissue content (Fig 3.4D), and that inability of roots to retain K^+ resulted in a loss of cell viability in sensitive genotypes (Figs 3.3, 3.4). Moreover, the cell viability loss was more severe in the elongation zone tissues which also showed a significantly higher K^+ loss as compared to mature root tissues (Fig. 3.5). In this context, the current results are consistent with previous studies which reported a reduction in K^+ uptake in response to waterlogged conditions at whole plant level (Colmer and Greenway, 2010; Elzenga and van Veen, 2010; Zeng et al., 2014) and exogenously applied K^+ showed beneficial effects on plant performance and alleviated the hostile effects of waterlogging (Ashraf et al., 2011; Wang et al., 2013). Taken together, the data reported in the present study implicates cytosolic K^+ retention as a key determinant of plant adaptive ability to hypoxia stress.

Interestingly, hypoxia-induced K^+ efflux from the elongation zone increased in the first hours after stress onset but was then reduced to zero (Figs. 3.5A, 3.7A). These results may be indicative of the signaling role of K^+ in this tissue. It was suggested that the transient decline in the cytosolic K^+ pool might help plant survival from energy crises by 'shutting down' many energy-dependent processes such as protein synthesis and entering into the defence mode (Demidchik et al., 2014; Shabala et al., 2014). This concept developed largely for salinity stress

signalling (Shabala, 2017) appears to be valid for hypoxia signalling as well. Under this scenario, a quick loss of K^+ from the cytosol and associated reduction in the enzymatic activity would serve as a push to redirect available energy from the energy expensive processes of structural protein synthesis, to defense-related processes, such as the prevention of cytosolic acidification, detoxification of ROS and production of molecular chaperones (Shabala et al., 2014; Zeng et al., 2014). This approach may significantly reduce the consumption of ATP and thus, increase the overall fraction of available ATP pool to fuel the continued operation of H^+-ATPase to sustain MP under O_2 limited conditions. Once the signalling is over and expression of the appropriate genes has been triggered, K^+ efflux from the elongation zone is reduced to zero (at 24 h and 48 h time points; Fig 3.4 A), to prevent further depletion of K^+ resources and a loss of cell viability.

The kinetics of hypoxia-induced K^+ fluxes across the plasma membrane in mature zone is drastically different from the elongation zone. Here, a time-dependent progressive decline in K^+ uptake is observed with K^+ uptake gradually turned into a net K^+ loss in sensitive genotypes, as hypoxia progressed (Fig 3.7A, 2.8). A strong positive correlation between the ability of mature zone cells to retain K^+, cell viability, and the overall waterlogging stress tolerance of a specific genotype, makes it possible to recommend using steady-state K^+ fluxes under these conditions (48 h hypoxia treatment; excised coleoptiles) as a physiological marker for breeding plants for waterlogging stress tolerance. Our results suggested that the K^+ flux measured in mature root zone both in control and treated plants was uniform and sensitive enough to discriminate between tolerant and sensitive cultivars (Fig. 3.5B, C). Given that measurements are conducted in a steady state, each of them requires only 1.5-2 min allowing ~30 to 40 specimens be measured in one hour. Thus, the suggested protocol may be applied to screen a large number of double haploid populations for developing molecular markers and mapping QTLs for waterlogging tolerance. It is worth noting that the root ability to retain K^+ has never been targeted in the breeding programs aimed to improve waterlogging stress tolerance in any species.

3.4.2 Regulation of voltage-gated outward-rectifying K^+ channels is critical for root K^+ retention

K^+ loss under hypoxic conditions may be triggered by a series of different potential factors such as (i) membrane depolarization and consequently channels opening (ii) changes to the

selectivity of K^+ in non-selective membranes (iii) K^+ leakage through ROS-stimulated channels (Shabala and Pottosin, 2014; Zeng et al., 2014). Up to now, there are 77 genes of conjectural K^+-permeable channels in *Arabidopsis* genome which are enabling K^+ transport across plant membranes. K^+-selective channel genes are comprised of nine Shaker-type channels (Véry and Sentenac, 2002; Demidchik et al., 2014). Two of them - SKOR (located in the stellar tissue) and GORK (located in root epidermis) – are classified as outward-rectifying K^+ channels. SKOR facilitates K^+ release from the xylem parenchyma cells to the xylem vessels whereas GORK is playing its role in the leakage of K^+ into external media (Demidchik et al., 2014).

Earlier pharmacological studies suggested that hypoxia-induced changes in K^+ fluxes could potentially be mediated by both voltage-dependent K^+-inward (KIR) and K^+-outward (KOR) rectifying channels (Pang et al., 2006). However, strong depolarization of membrane potential under hypoxic conditions reported in this work (Fig 3.9) makes the involvement of KIR channels (such as AKT or KAT) thermodynamically impossible. Indeed, the reported values of MP in Fig 3.9 in hypoxia-treated roots are around -90-95 mV. Assuming cytosolic K^+ concentration being at least 100 mM (Dreyer and Uozumi, 2011), for passive (channel-mediated) K^+ uptake [K^+] in the bath solution should exceed 2 mM, while in our case it was only 0.2 mM. This point out the GORK channel as a most likely candidate to mediate the observed effects of hypoxia in K^+ transport in barley roots. Indeed, hypoxia-induced K^+ loss and MP showed a strong association; less negative values of MP were accompanied by the severe loss of K^+ in a very clear and genotype-specific manner (Fig 3.5A, B; Fig 3.9A, B). Also, the genotypes or tissues with more negative values of MP and less K^+ loss showed better morphological growth and better cell viability when exposed to hypoxia or waterlogging (Figs 3.2, 3.4A). Importantly, in addition to MP depolarization, GORK channels are also known to be activated by ROS (Demidchik et al., 2010; García-Mata and Lamattina, 2010) which can be rapidly produced under hypoxia stress (Rhoads et al., 2006).

The relative expression of *GORK* decreased in the mature zone; however, a 10-fold increase was found in the elongation part when exposed to hypoxia (Fig. 3.10B), potentially explaining higher sensitivity of this part to hypoxia, as evidenced by viability staining (Fig 3.3). Once again, this indicates that GORK channel is most likely the candidate to control stress-induced K^+ signalling and homeostasis in hypoxia-treated roots. The difference in the expression levels of *GORK* in mature and elongation zone were mirrored by the changes of K^+ effluxes when measured from different root zones of barley in hypoxic conditions (Figs. 3.5A,

3.7A). The present findings are in full agreement with a recent report in which *Arabidopsis* showed a waterlogging tolerant phenotype after knocking out GORK channels (Wang et al., 2016).

3.4.3 H^+-ATPase activity and/or expression determines GORK operation

Oxygen plays a key role in the process of efficient production of ATP in aerobic organisms (Voesenek et al., 2006). Oxygen-deficient conditions lead towards energy crises by limiting O_2 availability for ATP production which results in a limited supply of energy to fuel H^+-ATPase pumps which enable H^+ extrusion (Bailey-Serres and Voesenek, 2008; Licausi and Perata, 2009; Shabala et al., 2014). H^+ pumps are the main electrogenic systems responsible for maintaining negative membrane potential values at the root plasma membrane (Teakle et al., 2013; Shabala et al., 2014). At the same time, they are also major consumers of ATP. In this study, the role of H^+-ATPase as a key regulator of intracellular K^+ homeostasis was shown directly in experiments by modifying O_2 transportation to the shoot in experiments with excised coleoptiles (that operates as a snorkel under oxygen-limited conditions). The intact roots showed a significant higher H^+ efflux as compared to excised roots when measured from the mature root zone of barley under hypoxia stress (Figs. 3.5; 3.7). The barley cultivars showed a strong correlation between H^+ flux and PM depolarization when measured from intact roots (Figs; 3.5; 3.9) which were further confirmed by the significant down-regulation of H^+-ATPase transcript levels. H^+ efflux was strongly inhibited by vanadate (Fig. 3.9C, D), a known inhibitor of PM H^+-ATPase. Our results of reduction in the H^+-ATPase expression are consistent with previously reported results of significant reduction in the H^+-ATPase activity just after 2 h of anoxic treatment in pea epicotyls (Koizumi et al., 2011). Potentially, coleoptile excision may also interfere with the root K^+ uptake and/or retention due to disrupted K^+ cycling between shoots and roots. However, our early experiments showed that effects of coleoptile excision on root K^+ fluxes and MP was identical to that achieved by full coleoptile submergence (Zeng et al., 2014) making the above scenario unlikely.

The two categories of H^+ pumps are the tonoplast-located vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-inorganic pyrophosphatase (V-PPase). These pumps show important roles for the accumulation of key ions into the vacuoles by generating electrochemical H^+ gradient through vacuolar membranes (Sze et al., 1992). V-ATPase pump plays a crucial role in avoiding the cytosolic vacuolar acidification under oxygen-deficient conditions (Greenway

and Gibbs, 2003; Koizumi et al., 2011). Insufficient supply of oxygen reduced the PM and V-H^+-ATPase activities (Zhao *et al.* 2012). In the state of an inadequate V-ATPase activity, the vacuolar H^+-pyrophosphatase (V-PPase) performs as an additional or alternative powerhouse of the tonoplast (Greenway and Gibbs, 2003). It was suggested that a shift from V-ATPase to V-PPase-driven H^+ extrusion is useful to roots with limited oxygen availability (Greenway and Gibbs, 2003). The breakdown of PPi is more favourable to ATP levels when ATP availability and cytoplasmic pH drop down due to hypoxia (Felle, 2005). There are three H^+-pyrophophatase genes have been labelled in barley. The first two are *HVP1* and *HVP10*, which are more reactive to abiotic stresses. Both displayed higher levels of gene expression in roots of barley (Fukuda *et al.* 2004) but they have different gene expression patterns. A third V-PPase gene, *HVP3*, which is hypothetically is not associated with abiotic stresses and involved in proton pumping only during seed development (Wang *et al*. 2009).

In this study, two V-PPase encoded genes *HVP1* and *HVP10* transcript levels were regulated differently depending on the type of tissues. A substantial upregulation of *HVP10* expression was measured from the elongation zone which was more sensitive to hypoxia as compared to the mature zone where it showed a reduction in the transcript level (Fig. 3.10D). In the mature zone, that under natural conditions has better access to oxygen (Zeng et al., 2014) and thus is capable of maintaining higher H^+-ATPase activity (as judged by more negative MP; Fig 3.9), the upregulation of V-PPase may be not required. The opposite scenario occurs in the elongation zone. In this context, our results are consistent with previous reports (Carystinos et al., 1995; Harada et al., 2007) of a massive increase in the transcript level of V-PPase after plant exposure to anoxic conditions followed by its decrease upon return to the well-aerated state. It should be commented that V-PPase activity shows a strong K^+ dependence (Obermeyer et al., 1996). Given much poor K^+ retention ability in the elongation zone (Fig 3.7) one can assume that the efficiency of V-PPase operation in this tissue would be reduced under hypoxic conditions. Thus, the observed increase in the transcript level of *HVP10* may be taken as a plant's attempt to compensate for decreased V-PPase activity by increasing the number of functional units, to protect this zone from cytosolic acidosis under hypoxia stress.

Chapter 4

Cell-based phenotyping reveals QTL for membrane potential maintenance associated with hypoxia and salinity stress tolerance in barley

4.1 Introduction

Waterlogging is one of the major abiotic stresses limiting agricultural production around the globe (Setter and Waters, 2003). It imposes several limitations on plants during their life span (Bailey-Serres and Voesenek, 2008; Shabala and Pottosin, 2014). Among them, the major constraint that a plant exposed to waterlogging is either a complete unavailability or an inadequate supply of oxygen to submerged organs of flooding sensitive species (Armstrong and Drew, 2002). As a result, the transport of nutrients from roots to shoots is severely disturbed under waterlogged conditions (Smethurst et al., 2005; Colmer and Voesenek, 2009), which consequently affects plant growth and yield (Malik et al., 2001; Colmer et al., 2011). Salinity is another limiting factor for crop production. According to FAO (2008), almost 800 million hectares of the global land area are affected by salinity which accounts for more than 20% of the irrigated land area (Yamaguchi and Blumwald, 2005). Under saline conditions, excessive accumulation of Na^+ and Cl^- results in their toxicity. Salinity stress also imposes osmotic and oxidative stress and interferes with the uptake and retention of other mineral elements such as K^+ (Benito et al., 2014). Taken together, these factors lead to a disturbance of plant metabolism, reduced growth rates and plant yield. To meet the target of more than 70% increase in food production by 2050 (Garnett et al., 2013), it is important to improve the plant's tolerance to cope with different abiotic stresses, including waterlogging and salinity.

Barley is considered to be a waterlogging sensitive (Zhou et al., 2012) and moderately salt tolerant cereal (Ullrich, 2002; Munns et al., 2006), although it shows significant variation over genotypes in waterlogging (Takeda and Fukuyama, 1986; Zhou, 2010) and salinity tolerance (Slavich et al., 1990; Jaradat et al., 2004). Many QTLs have been reported for waterlogging and salinity tolerance based on different physiological and agronomic traits. For waterlogging tolerance, QTL mapping was done targeting aerenchyma formation (Mano and Omori, 2009; Zhang et al., 2015), root porosity (Broughton et al., 2015), grain yield (Zaidi et al., 2015), leaf chlorosis (Li et al., 2008; Zhao et al., 2012; Ma et al., 2015) and plant biomass (Zhang et al., 2013) as the whole-plant based phenotypic traits. Several QTLs have also been

identified for salinity tolerance by using many whole-plants based phenotypic indices such as shoot sodium content (Rivandi et al., 2010; Shavrukov et al., 2010), intercellular CO_2 concentration (Liu et al., 2017) and germination rate (Mano and Komatsuda, 2002). However, none of these findings led to any major progress in creating stress-tolerant cultivars. Several reasons may explain this (Arzani and Ashraf, 2016). First, the statistical testing of null hypotheses (for example no QTL) is deeply embedded in the probability theory and conditions that create error variance leads to threats to statistical conclusion validity. The LOD threshold value for avoiding a false positive with a given confidence, say 95%, depends on the number of markers and the length of the genome. As a consequence, the literature describing QTL analyses might contain false-positive QTLs at the too high rate. The second major reason is the genotype (QTL) by environment interaction which often confounds with main effects of a QTL. This is specifically true for all field-based studies. Next, the quantitative genetic models are often based on certain (unrealistic) assumptions and also have strong background dependency.

From a physiological point of view, the major shortfall is that in nearly all cases the above phenotyping has been conducted at the whole-plant level, so each of the measured traits was conferred by multiple (and often unrelated) contributing mechanisms. As a result, multiple QTLs have been reported for each of these traits. For example, fourteen QTLs were associated with leaf chlorosis on chromosomes 1H, 2H, 3H, 4H, 5H, 6H, 7H for waterlogging tolerance (Li et al., 2008; Xu et al., 2012; Zhou et al., 2012) and ten QTLs associated with plant height on chromosomes 1H, 2H, 4H, 5H, 7H for yield component (Li et al., 2005; Xue et al., 2010; Chutimanitsakun et al., 2011). The second reason is that very often the phenotypic indices used are not directly related to the mechanisms targeted and are, therefore, misleading. For example, measuring whole-shoot Na^+ content in all studies (Genc et al., 2007; Haq et al., 2014; Tounsi et al., 2016) fails to account for differential ability of plants to sequester Na^+ in leaf vacuoles; the trait considered to be the most crucial to confer salinity tissue tolerance. As a result, the amount of Na^+ measured in the shoot will be the same for highly salt-sensitive species such as pea or rye and highly salt-tolerant halophyte species, but the impact on growth will be drastically different. Thus, it appears that the real progress in plant breeding can be achieved only when plant phenotyping will directly target a contributing mechanism. This can be achieved only when such phenotyping is conducted at the cellular level.

Waterlogging and salinity tolerances are complex traits that are conferred by numerous physiological mechanisms (Jackson et al., 2009; Qiu et al., 2011; Shabala et al., 2016). Amongst these, the plasma membrane (PM) H^+-ATPases play a central role in cell ionic homeostasis and stress signalling and adaptation. Channel-mediated root nutrient acquisition depends on the electric potential difference (membrane potential) across the PM, which is controlled by the H^+-ATPase activity (Palmgren and Nissen, 2011). H^+ pumps also create a proton motive force for the secondary active ion transport (Shabala et al., 2016). The strong correlation between root plasma membrane H^+-ATPase activity and an overall salinity stress tolerance was found in many species (Chen et al., 2007; Bose et al., 2014; Lei et al., 2014). The same is true for waterlogging stress. Most of the membrane transporters are voltage-gated in nature, and the PM is significantly depolarized (typically by 40 to 70 mV) under oxygen-limited conditions due to insufficient ATP availability (Teakle et al., 2013; Zeng et al., 2014).

H^+-ATPase-mediated maintenance of a highly negative membrane potential is one of the key elements of the maintenance of intracellular K^+ homeostasis. Potassium (K^+) is an essential and most abundant nutrient which plays significant roles in plant growth. K^+ is involved in the cell turgor pressure maintenance, cell elongation, stress signalling and osmoregulation (Dreyer and Uozumi, 2011; Shabala and Pottosin, 2014). Stress-induced membrane depolarization activates outward-rectifying K^+ efflux channels (GORK in Arabidopsis), resulting in a massive K^+ loss under both hypoxia (Elzenga and van Veen, 2010) and salinity stress conditions (Chen et al., 2005), and leading to a significant reduction in plant K^+ content (Smethurst et al., 2005; Board, 2008). This decline in K^+ content results in severe yield penalties (Drew and Sisworo, 1979; Robertson and Vitousek, 2009) and, in extreme cases, in the loss of the cell viability (Shabala et al., 2016). The plant's ability to survive under waterlogged and saline conditions could be improved by improving its K^+ retention capacity (Wang et al., 2013). Interestingly, plants often respond to salinity stress by an increase in the GORK transcript level (Adem et al., 2014; Chakraborty et al., 2016) suggesting that it is a post-translational regulation of GORK channel that is crucial for adaptive responses to stress. As mentioned above, voltage gating is arguably the most essential factor in this regulation. Thus, finding the QTL responsible for such gating may open a novel and previously unexplored avenue for improving abiotic (salinity and waterlogging) stress tolerance via enhanced K^+ retention.

In this study, we have adopted a new method to phenotype plants at the single-cell level, to account for the tissue-specific expression of transporters, and identify a QTL responsible for

the maintenance of negative membrane potential under hypoxic conditions. This method relied on using the microelectrode MIFE technique and has been applied to screen 150 barley double haploid (DH) lines from a cross between TX9425 and Naso Nijo under hypoxia (waterlogging). Analyses were conducted to identify the linkage between this trait and waterlogging/salinity tolerances. For the first time in the literature, we report a major QTL for the membrane potential. This finding may open new avenues for future breeding programs to develop more tolerant varieties.

4.2 Materials and Methods

4.2.1 Plant material

A total of 150 DH lines from a cross between TX9425 and Naso Nijo (Xu et al., 2012) were used in this study for membrane potential measurements. TX9425 is Chinese, two-rowed barley variety which is tolerant to waterlogging and salinity (Pang et al., 2004; Zhou et al., 2007) and shows a few exceptional agronomic characteristics (Wang et al., 2010) and resistance to some diseases (Li et al., 2009; Li and Zhou, 2011). Naso Nijo is a Japanese malting barley variety with good agronomic characteristics but is sensitive to both waterlogging (Pang et al., 2004) and salinity (Xu et al., 2012).

Seeds were surface sterilized with 10% commercial bleach (NaClO 42 g L^{-1}; Pental Products, Shepparton, Australia), thoroughly rinsed with tap water for at least 30 min and then grown in wet paper rolls with basic salt media (BSM) solution (0.5 mM KCl + 0.1 mM $CaCl_2$, pH 5.6) in the dark for 3 days at room temperature (25 ± 1°C). Two treatments were used in the present experiment: (1) control (BSM, aerated); and (2) hypoxia (BSM solution made with 0.2% agar and bubbled with N_2 gas). For the treatment with agar, the stagnant solution was prepared by adding agar (Cat. No. LP0011, Oxoid, Hampshire, UK) to the BSM solution at a ratio of 0.2% (w/v) and boiled, then cooled overnight to room temperature with magnetic stirring to prevent lump formation. The agar solutions were pre-bubbled with high purity N_2 (Cat. No. 032G, BOC Gases, Hobart, Australia) for at least 1 h before being used in the experiment.

4.2.2 Evaluation of the DH lines for waterlogging and salinity tolerance

All the details related to waterlogging and salt tolerance evaluation experiments are given in our previous publication (Xu et al., 2012). In brief, for waterlogging, tolerance evaluation DH lines generated from a cross between TX9425 and Naso Nijo were subjected to waterlogging for nine weeks until sensitive lines died. A collective scoring system was used, with scoring index 0 indicating no damage and index 10 given to dead plants. Plants with scores 0-5 showed various levels of chlorosis and those with scores 6 or above showing a substantial percentage of necrotic leaves, under waterlogged conditions. To evaluate salt tolerance, seeds of the DH lines were sown in 40-L containers filled with a pine bark/loam-based potting mix with premixed slow release fertilizer. After germination (7 days after sowing) 200 mM NaCl treatment was applied and maintained until data collected. Salt tolerance was assessed by combining scores for leaf chlorosis and plant survival after 7 weeks of sowing and conducted in a similar way to the one described above for waterlogging.

4.2.3 MIFE ion flux measurements

The net flux of K^+ was measured from barley roots with a length of 60 ± 10 mm by using a non-invasive ion flux measurement technique, MIFE (University of Tasmania, Hobart, Australia). The theory of MIFE measurements and other details of calibration and fabrication related to ion-selective microelectrode are given in our previous publications (Shabala et al., 2010; Wu et al., 2015). In brief, borosilicate microelectrodes with the tip diameter of 2-3 µm were filled with corresponding backfilling solution. Electrode tips were then front-filled with a suitable Liquid Ion Exchanger (LIX) (Fluka Catalogue no. 60031 for K^+ and 95297 for H^+). The electrodes were mounted on a 3D-micromanipulator and calibrated with a set of pH and different K^+ standards by using three-point calibration. Electrodes were then positioned 40 µm away from root surface and placed on the same plane 2-3 µm to each other. During the measurement of fluxes, microelectrodes were moving between two positions 40 µm close to root epidermis and away 90 µm in a (10 s cycle) square-wave manner. The potential difference between these two points was recorded by a MIFE CHART software and converted into electrochemical potential difference by utilizing the cylindrical diffusion geometry (Newman, 2001).

Prior to measurement a 3-day old seedling was taken from a paper roll and immobilized horizontally by plexiglass partitions 2 mm above from the surface of the chamber. The measuring chamber was filled with hypoxia solution while coleoptile being above the surface

of the solution. Seedlings for control were well aerated during the period of treatment; whereas hypoxia (0.2% agar) treated seedlings were kept in stagnant conditions for 48 h. The seedlings were then placed in Faraday cage for MIFE measurements. Net ion flux was measured for 10 min; the steady state flux was attained by averaging values of the last 5 minutes flux.

4.2.4 Membrane potential measurements

Membrane potential (MP) values were measured from root epidermal cells of intact barley seedlings. Conventional microelectrodes (Harvard Apparatus) were filled with 1 M KCl and connected to MIFE electrometer via Ag/AgCl half-cell. During membrane potential measurement, the microelectrode with a tip diameter of 0.5 µm was manually impaled into the epidermal cells of the mature root zone (5 mm from shoot base) using a functioned 3D-micromanipulator (MHW-4, Narishige, Tokyo, Japan). Membrane potential values were recorded by the MIFE CHART software for at least two minutes after stabilization (Newman et al., 2001).

Before measurement, a 3-day old seedling was taken from a paper roll and mounted in a vertical chamber and treated then with hypoxia solution. The measuring chamber was filled with hypoxia solution with the coleoptile being above the surface of the solution. Roots were kept under stagnant conditions for 48 h. The seedlings were then placed into the Faraday cage for membrane potential measurements. For each DH line, membrane potential values were measured from the roots of 5-6 individual seedlings after 48 h of treatment. At least 4 measurements were taken from each seedling. The overview of the experimental procedure is further illustrated in Figure 4.1.

4.2.5 Map construction and QTL analysis

Genomic DNA of the DH population was extracted from the leaf tissue of four-week-old seedlings. A total of 28047 DArT and 8928 SNP markers were used for genotyping. After removing markers with greater distortion and missing data, 4788 markers were chosen for map construction. A new genetic map of the DH population was constructed using the software package JoinMap 4.0 (Van Ooijen, 2006). A QTL analysis was conducted using the software package MapQTL 6.0 (Ooijen and Kyazma, 2009). Interval mapping (IM) was firstly used to detect the major QTL. The nearest marker at the QTL from IM was chosen as a cofactor in the multiple QTL models (MQM). The logarithm of the odds (LOD) threshold values applied to

declare the presence of a QTL were estimated by performing the genome-wide permutation tests implemented in MapQTL version 6.0 using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold around 3.0. To determine the effects of physiological traits on waterlogging and salinity tolerance, QTL for both waterlogging and salinity tolerance were re-analysed by using various physiological traits as covariates. Maps showing the QTL position and LOD values were generated using MAPCHART (Voorrips, 2002).

4.3 Results

4.3.1 Membrane potential values of parents and DH lines under hypoxia stress

The protocol for membrane potential measurements from barley roots via microelectrode MIFE technique is shown in Fig. 4.1. Both parent cultivars showed a significant difference in membrane potential values when measured from epidermal root cells of barley after 48 h of hypoxia stress. Under hypoxia stress, membrane potential values of the waterlogging tolerant parent TX9425 were significantly more negative (-125.3 ± 3.3 mV) than of sensitive parent Naso Nijo (-83.4 ± 2.9 mV) (Table 4.1). The DH lines from the cross between TX9425 and Naso Nijo also showed a significant difference in values of membrane potential when exposed to hypoxia for 48h. Fig. 4.2 shows the frequency distribution of waterlogging tolerance based on the membrane potential values and net K^+ flux under hypoxia stress. A continuous distribution was found for membrane potential with values ranging from -41 to -138 mV (Table 3.1). Analysis of variance (ANOVA) for membrane potential showed a significant difference ($P < 0.001$) between DH lines under hypoxia stress (Table 4.2).

Table 4.1 Effects of hypoxia (N_2 bubbled 0.2% agar) stress on membrane potential values of parents and DH lines. Data are mean values ± S.E.

Cultivars	Membrane potential (mV)
TX9425	-125.33 ± 7.34
Naso Nijo	-85.42 ± 6.96
DH lines average	-91.17 ± 14.54
DH lines range	-40.97 - -137.52

4.3.2 QTL for membrane potential

One major QTL for membrane potential was identified on chromosome 2H which was designated as *QMP.TxNn.2H*. This QTL was detected close to the 8613801D2 marker at the position of 8.85 cM and explained 22% of the phenotypic variation (Table 4.3). The position of the QTL identified in this study was the same as that for waterlogging tolerance (Xu et al., 2012) (Fig. 4.3). No significant QTL was identified for net K^+ flux under hypoxia, although it showed significant effects between DH lines.

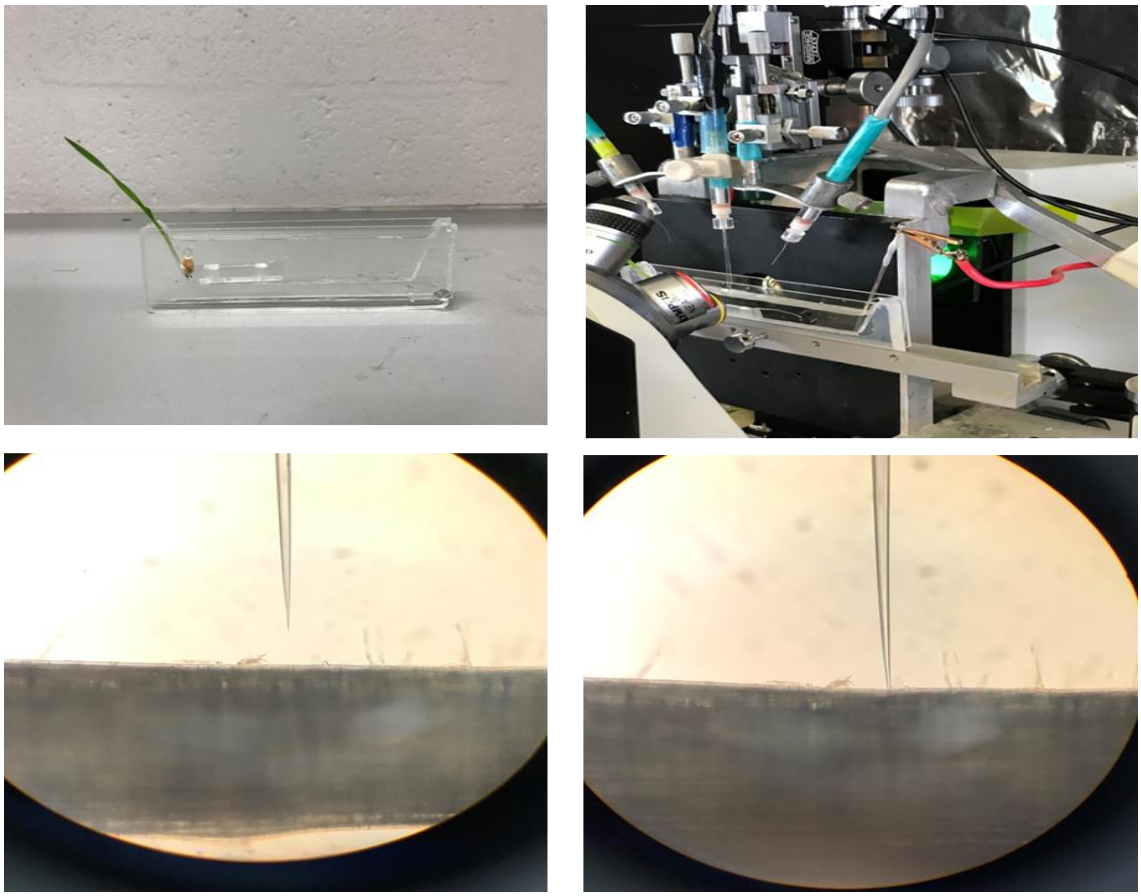

Fig. 3.1 Four steps of experimental procedure are illustrated. (A) Seedling is imbobilized in a vertical chamber and treated with hypoxia solution (N_2 bubbled 0.2% agar). (B) The vertical chamber is mounted in faraday cage for membrane potential measurements. (C) Electrode is positioned next to root epidermis. (D) Electrode is impaled into the root cell for membrane potential measurements.

Table 4.2 ANOVA of membrane potential values under waterlogging (hypoxia) stress.

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	376649.7	149	2527.85	40.23671	4.8E-192	1.23783
Within Groups	28271.02	450	62.82448			
Total	404920.7	599				

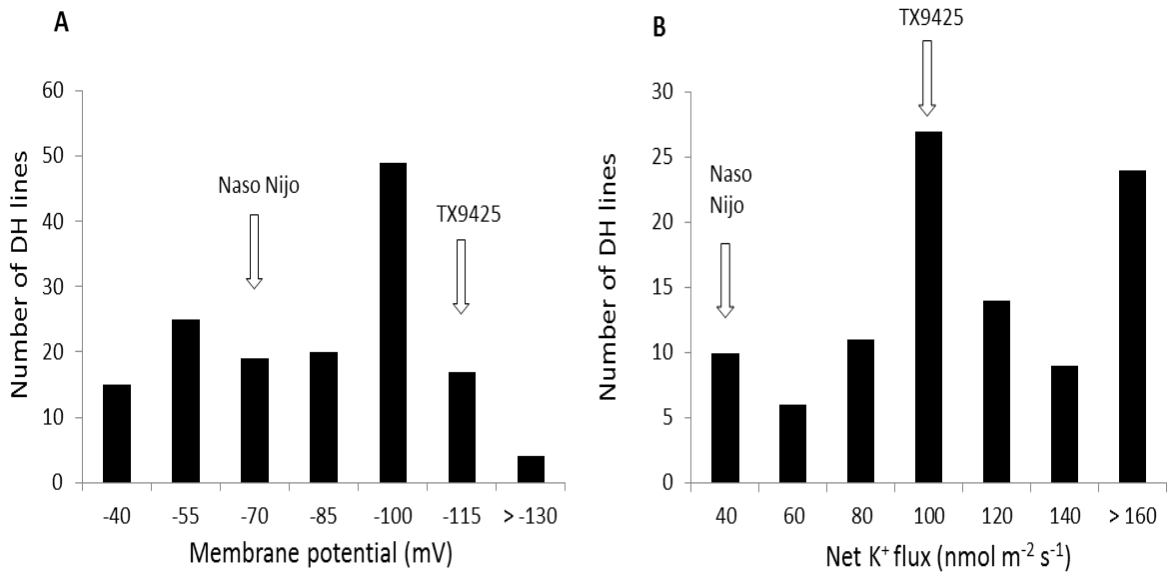

Fig. 4.2 The frequency distribution for membrane potential (MP) values (A) and net K^+ flux (B) under hypoxia (0.2% agar) stress of DH lines derived from a cross of TX9425 and Naso Nijo.

Table 4.3 QTL on 2HS for membrane potential, salt and waterlogging tolerance detected in the DH population of TX9425 × Naso Nijo.

Traits	Linkage group	Nearest marker	Position (cM)	LOD	R^2 (%)	Additive effect	Co-variate
MP	2H	8613801D2	8.85	6.89	22	-11.9	
	2H	8613801D2	8.85	6.2	19.5	-12.4	Waterlogging
	2H	8613801D2	8.85	1.99	5.7	-9.4	Salt
Waterlogging	2H	3258828D2	9.21	7.61	21	0.73	
	2H	3258828D2	9.21	5.83	18.4	0.76	MP
Salt	2H	3259260S2	7.79	32.79	63.7	2.40	
	2H	3259260S2	7.79	26.29	50.8	2.49	MP

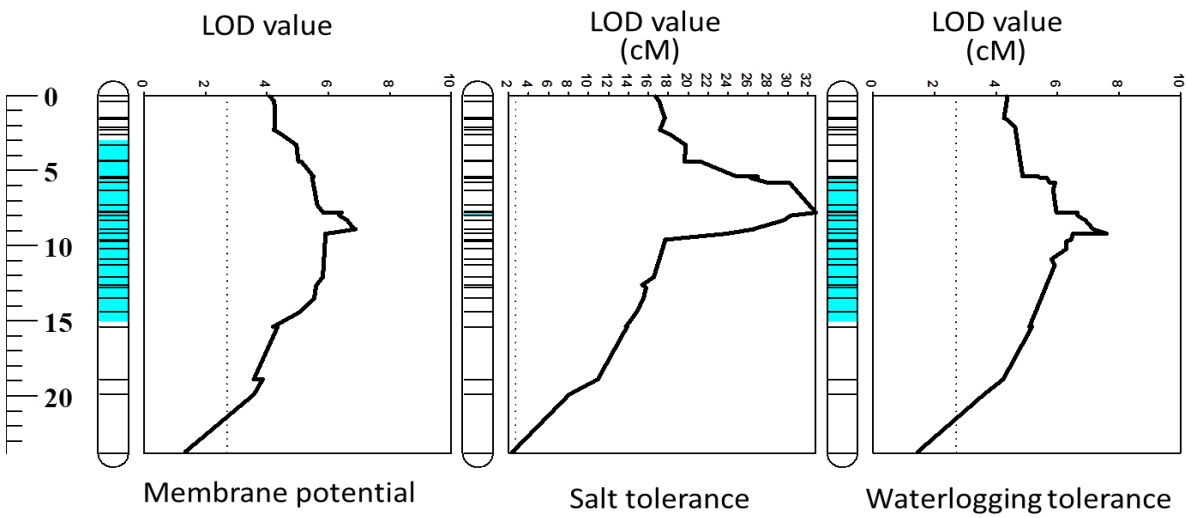

Fig. 4.3 QTL for membrane potential, salt and waterlogging tolerances on 2HS. The figures related to salt and waterlogging tolerance incorporate data published by Xu et al. (2012). The full length of chromosome 2H is also displayed in this study.

4.3.3 Contribution of membrane potential to waterlogging and salt tolerance

MP showed a significant ($P<0.05$) correlation with waterlogging tolerance (Fig. 4.4A). This is further confirmed by QTL analysis for waterlogging tolerance using MP as a covariate. As shown in Figure 4.5A, the LOD value of the QTL on 2H for waterlogging tolerance showed a slight reduction when MP was used as a covariate. The percentage of the phenotypic variation (R^2) determined by the QTL also showed a slight reduction, from 21.0% to 18.4% (Table 4.3). MP also showed a close and significant correlation ($P<0.001$) with salt tolerance (Figure 4B). Correlation between MP and salt tolerance is higher than the correlation between MP and waterlogging tolerance. When MP was used as a covariate, LOD value and R^2 of the QTL for salt tolerance reduced from 32.8 to 26.3 and 63.7 to 50.8, respectively (Fig. 4.5B; Table 4.3).

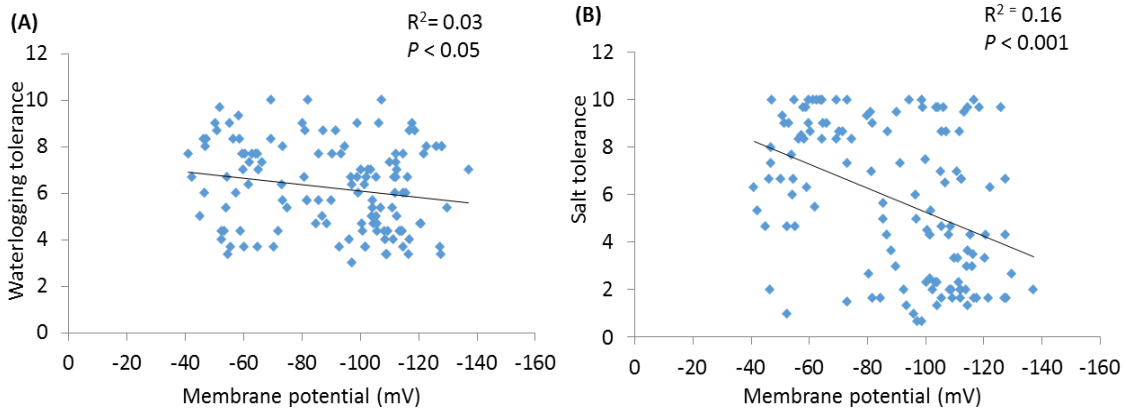

Fig. 4.4 Correlation between membrane potential and waterlogging tolerance scores (A) and between membrane potential and salt tolerance scores (B).

4.3.4 QTL for MP when using waterlogging and salt tolerance as covariates

The weak correlation with waterlogging tolerance and strong correlation with salt tolerance was further confirmed by reverse QTL analysis, i.e. analysis of QTL for MP using either waterlogging or salt tolerance as a covariate. When such analysis was conducted by using waterlogging damage scores as a covariate, only slight reductions in both LOD and R^2 of the QTL for MP were found while the QTL for MP became insignificant when salt tolerance scores were used as covariates (Fig. 4.6).

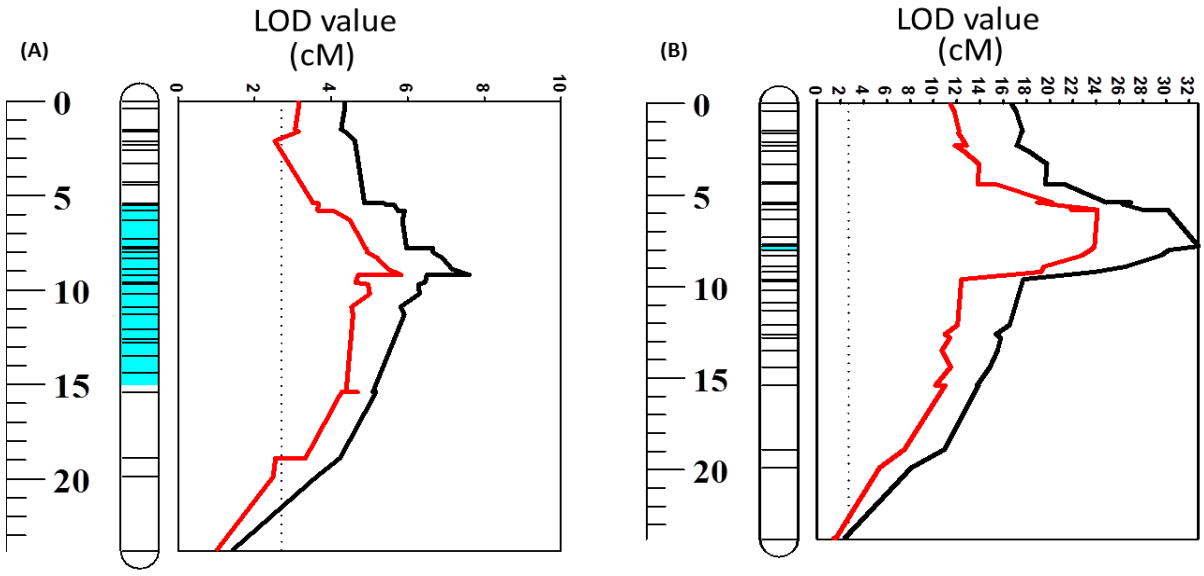

Fig. 4.5 QTL associated with waterlogging tolerance (LOD values) on 2HS (A) and QTL associated with salt tolerance (LOD values) on 2HS (B). Black line: LOD value of original QTL; Red line: LOD value of QTL when membrane potential is used as a covariate.

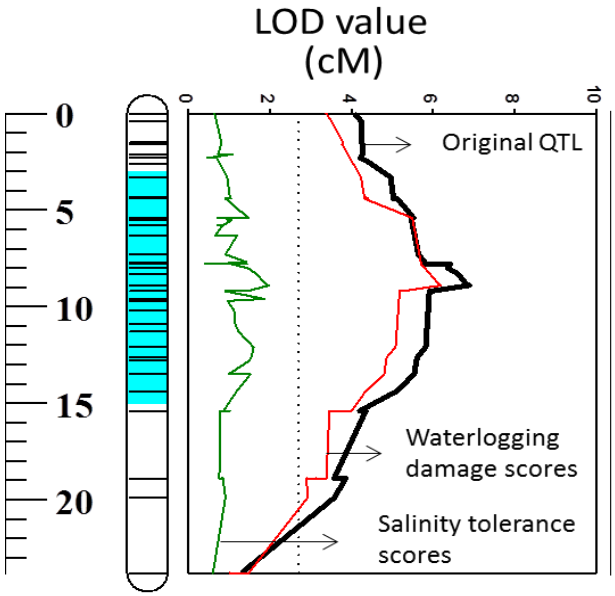

Fig. 3.6 QTL associated with membrane potential (LOD values) on 2HS. Black line: LOD value of original QTL; Red line: LOD value of QTL when waterlogging damage scores are used as a covariate; Green line: LOD value of QTL when salinity tolerance scores are used as a covariate.

4.4 Discussion

Tolerance to abiotic stresses is an important breeding objective. Great efforts have been made to identify mechanisms conferring waterlogging/salinity tolerance and finding QTL for the tolerance using different screening systems (Aslam et al., 1993; Mano and Takeda, 1997; Foolad et al., 2001; Lee et al., 2006; Lee et al., 2007; Chen et al., 2008; Chen et al., 2008; Xue et al., 2009; Fan et al., 2015). However, the practical outcomes are still disappointingly small. Both waterlogging and salinity tolerances are highly complicated traits that are controlled by many different mechanisms. Direct selection of the overall tolerance is very hard thus breeders rely on molecular markers linked to the tolerance. Most QTL identified for waterlogging/salinity tolerance are based on plant survival rate, plant healthiness and leaf chlorosis under stress (Li et al., 2008; Xue et al., 2010; Xu et al., 2012; Zhou et al., 2012; Ma et al., 2015; Zhang et al., 2016). While these traits are convenient for high throughput screening, they are not directly related to the mechanisms conferring the tolerance. As a result, fine mapping of these QTLs to provide reliable markers to breeders is very difficult, even if possible in principle due to the very large number of QTLs involved.

Much more promising is an approach when specific QTLs are linked directly with appropriate mechanisms. Since most of the mechanisms are expected to be controlled by just one or two QTLs, these are much easier to fine map. A good example of this success is for barley waterlogging tolerance, the major QTL for waterlogging tolerance on 4H (Li et al., 2008; Zhou, 2011; Zhou et al., 2012) is due to the formation of aerenchyma under stress which is controlled by a single major QTL (Broughton et al., 2015; Zhang et al., 2016; Zhang et al., 2017) and the gene has been fine mapped to a < 2 cM region with closely linked markers being available for breeders to use.

The plasma membrane (PM) is responsible for the maintenance of ionic and electric gradients between the cytosol and external media and thus essential for intracellular ionic homeostasis. It is also an important component of the signal transduction in plants under stress conditions (Kim et al., 2007). PM depolarization is one of the common features between salinity and waterlogging stresses, leading to a substantial disruption in the ionic homeostasis (Palmgren and Nissen, 2011; Shabala et al., 2016) which contributes to metabolic disturbances and ultimately determines the cell's fate. The electrogenic H^+-ATPase pumps play a significant role in maintaining the negative potential of the PM. Oxygen-limited conditions resulted in a

significant depolarization of the PM due to a huge decline in ATP availability to fuel H^+-ATPase. The PM is also depolarized as a result of massive Na^+ uptake under saline conditions. In our experiment, waterlogging/salt tolerant variety, TX9425, showed a much better ability to maintain MP under hypoxia stress than waterlogging/salt sensitive variety, Naso Nijo (Table 4.1). The DH population from these two varieties showed a wide range of segregation (Fig. 4.2) and a major QTL (*QMP.TxNn.2H*) for MP (Fig. 4.3) was identified. This QTL is located on the short arm of chromosome 2H and explained 22% of phenotypic variation (Table 4.3). The fact that only one single major QTL was identified in this population makes it easier to further fine map the gene.

A large number of QTLs for different stress tolerances were reported at this position (Zhang et al., 2017), which include waterlogging (Zhou, 2011; Xu et al., 2012), salinity (Xu et al., 2012) and drought (Fan et al., 2015) with some being identified from the same DH population used in this study. Importantly, all these stresses are known to affect H^+-ATPase activity and depolarize the plasma membrane (Shabala et al., 2014). *Arabidopsis* mutants lacking an H^+-ATPase isoform showed increased sensitivity to salt and accumulate higher concentrations of Na^+ in leaves compared to wild-type plants (Vitart et al., 2001). On the contrary, expressing *Arabidopsis thaliana* V-ATPase subunit C in barley improves plant performance under saline condition by enabling better osmotic adjustment (Adem et al., 2017). When comparing MP with waterlogging/salt tolerance scores from the same population, MP showed significant correlations with both waterlogging and salinity tolerance (Fig. 4.4). Further QTL mapping of different traits was conducted using other related traits as covariates, which has been proved to be effective in confirming the relationship between different traits (Fan et al., 2015). When MP was used as a covariate the LOD value and R^2 of the QTL on 2H for both waterlogging and salt tolerances reduced with less effect on waterlogging tolerance, confirming the weak linkage between MP and waterlogging tolerance and greater contribution of MP to salt tolerance (Fig. 4.5 and Table 4.3). The reason for the different contribution of MP to the different stresses may be related to the difference in the principal causes for membrane depolarization upon low oxygen and salinity (e.g. compromised mitochondrial operation for the former and massive influx of Na^+ for the latter).

It is not clear at this stage what specific gene contributes to the better maintenance of higher MP values in hypoxia-affected barley roots. The nearest marker of the QTL detected in this study was located around 8.28 cM. No known subunits of H^+-ATPase appear to be present

in the vicinity of the reported marker suggesting that it was a *regulation* rather than the physical presence of the H^+-ATPase protein matter for MP maintenance. It was previously shown that the plant's ability to maintain negative MP was not attributed to changes in H^+-ATPase transcripts or the actual amount of protein (reviewed in Shabala et al. 2016) but rather regulated by the post-translational modifications. This regulation may occur via multiple pathways; one of them involves hormonal signalling. For example, it is known that ABA dephosphorylates the penultimate Thr residue on the H^+-ATPase, resulting in deactivation of the pump (Hayashi et al., 2014), and the protein kinase PKS5/CIPK11, an important element of ABA signalling cascade (Lumba et al., 2014), reducing the activity of the H^+-ATPase (Fuglsang et al., 2007). Additionally, an overexpression of a tomato 14-3-3 homologue (GRF9) resulted in an increased H^+-ATPase activity (Hu et al., 2015). The above annotated contigs for the 3 to 13 cM region on chromosome 2H contains a large number of kinases and kinase-like proteins. Also, two genes related to energy metabolism were detected in the vicinity of the marker. These were ectonucleoside triphosphate diphosphohydrolase 5 (E-NTPDase) and dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex. E-NTPDases break down nucleoside tri- and diphosphates to nucleoside monophosphates and inorganic phosphate (Pi) and perform a wide range of functions. This includes purinergic signalling and control of the ATO concentration in ER and Goldgi lumen to regulate ATP-dependent processes (Massalski et al., 2015). It remains, therefore a task for future studies to answer the question which of them is responsible for regulation of H^+-ATPase activity under stress conditions.

In conclusion, a major QTL for membrane potential maintenance under hypoxia was identified using cell-based phenotyping involving microelectrode MIFE technique. The QTL is located at a similar position to that for waterlogging and salinity tolerance on chromosome 2H. MP showed a weak but significant linkage with waterlogging tolerance and a strong linkage with salt tolerance. As only one single major QTL was responsible for MP, this makes it easier to fine map this QTL and effectively use this gene in pyramiding different tolerance mechanisms in breeding programs.

Chapter 5

Identification of QTL related to ROS formation under hypoxia and their association with waterlogging and salt tolerance in barley

5.1 Introduction

Waterlogging is a worldwide constraint that considerably affects growth, development and the distribution of plant species. In waterlogged (hypoxia, anoxia) conditions, the main reason that caused the damage to plant growth is a limited supply of oxygen to the submerged tissues; particularly in roots (Colmer and Voesenek, 2009; Blom et al., 2011). Waterlogging stress dramatically reduces available oxygen concentration to below critical levels in roots due to low diffusion rate of gasses in soil and respiration of microorganisms (Colmer, 2003; Bhattarai et al., 2005). Soil waterlogging gradually leads to hypoxia and with time may even result in a complete absence of oxygen (anoxia), also prompted accumulation of CO_2 in the root zone (Ponnamperuma, 1984). Under these hypoxic and anoxic conditions, the oxygen deficiency limits the ability of plant roots to supply water and nutrients to shoots (Visser et al., 2000; Mustroph and Albrecht, 2003). To meet the target of more than 70% increase in food production by 2050 (Garnett et al., 2013; Smith, 2013), it is important to improve the plant's ability to cope with different abiotic stresses, including waterlogging.

Under waterlogged conditions, plants undergo multifaceted environmental perturbations including restricted availability of oxygen and carbon dioxide, excessive accumulation of ethylene (Voesenek and Sasidharan, 2013) and toxic elements in soil (Bailey-Serres and Voesenek, 2008; Lamers et al., 2013; Zeng et al., 2013). As a result, cells and tissues may become exposed to an oxidative stress. The plant responses to oxygen-deprived conditions also include elevated generation of reactive oxygen species (ROS) essentially as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2) (Blokhina et al., 2003; Bailey-Serres and Chang, 2005). All of these ROS can oxidize biological molecules, such as lipids, proteins, carbohydrates, nucleic acids and enzymatic activity, also triggering the breakdown of these molecules (Møller et al., 2007; Steffens, 2014). Under oxygen-limited conditions, ROS can be produced by multiple mechanisms in plant roots such as: by PM NADPH, mitochondrial dysfunction and due to metal ions (Suzuki et al., 2012; Shabala et al., 2014; Wudick and Feijó, 2014).

At the same time, ROS produced under oxygen-deprived conditions also plays significant roles as a signalling molecule in plants during a broad range of developmental and adaptive responses to waterlogging stress. There is the significant bulk of data accumulated over the years suggesting that ROS production, by either a plasma membrane (PM) NADPH oxidase and/or mitochondria, controls the plant adaptive responses under oxygen-limited conditions (Bailey-Serres and Chang, 2005; Rhoads et al., 2006; Foyer and Noctor, 2009). However, unbalanced production of ROS can damage cellular components and cause their dysfunction. To counter overproduction of ROS, plants use several enzymatic and non-enzymatic sources, major ones are superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidases (APX). Thus, due to the above mentioned damaging role of ROS overproduction in living tissues, plants ability to produce antioxidant enzymes generally correlated with susceptibility to environmental stresses, including waterlogging (Yordanova et al., 2004; Zhang et al., 2007; Hossain et al., 2011).

Many QTL associated with various environmental stresses have been reported in previous studies (Mano et al., 2006; Ahmed et al., 2012; Ma et al., 2015; Pushpavalli et al., 2015; Zhang et al., 2015; Huang et al., 2018), including barley. Several QTL have been identified for waterlogging tolerance in this species, based on different physiological and agronomic traits. This includes germination rate (Angaji et al., 2010; Parelle et al., 2010), total root dry weight (Naz et al., 2014), chlorophyll damage index (Bertholdsson et al., 2015), grain yield (Zaidi et al., 2015), leaf chlorosis (Li et al., 2008; Ma et al., 2015) survival rate (Ma et al., 2014), plant biomass indices (Li et al., 2008; Ballesteros et al., 2015) and photosynthetic characteristics (Pearson et al., 2011). However, each of these indices may be affected by numerous environmental constraints and therefore are not necessarily causally related to waterlogging stress, thus limiting their practical use. In recent studies, traits more directly related to waterlogging tolerance have been selected to identify QTL; this includes root porosity (Broughton et al., 2015), adventitious root development (Xu et al., 2017) and aerenchyma formation (Broughton et al., 2015; Zhang et al., 2016). However, to the best of our knowledge, no QTLs for traits associated with tissue-specific ROS productions under hypoxic conditions have been reported for any plant species, despite a very essential role of oxidative damage as a major constraint imposed by waterlogging stress.

In this study, 187 barley double haploid (DH) lines from a cross between TX9425 and Naso Nijo were screened ROS production under hypoxia (waterlogging) stress. For the first

time, we reported a major QTL both $O_2^{\cdot -}$ and H_2O_2. Analyses were also conducted to identify the linkage between this trait and waterlogging/salinity tolerances. This finding may open new avenues for future breeding programs to develop more stress tolerant varieties.

5.1 Material and methods

5.1.1 Plant material

Six barley (*Hordeum vulgare* L.) cultivars contrasting in waterlogging tolerance were used in the initial part of this study. Among these cultivars, CM72, TX9425 and Yerong are tolerant, Gairdner Franklin and Naso Nijo are sensitive to waterlogging (Pang et al., 2004; Zhou, 2011). Seeds were acquired either from China or the Australian Winter Cereal Collection Centre and reproduced in the field, using Tasmanian Institute of Agriculture (TIA) facilities in Launceston. For QTL analysis, data was collected from 187 double haploid (DH) lines originated from a cross between TX9425 and Naso Nijo. As mentioned earlier, TX9425 is Chinese, two-rowed barley variety which is tolerant to waterlogging and salinity (Pang et al., 2004; Zhou et al., 2007) and shows a few exceptional agronomic characteristics (Wang et al., 2010). Naso Nijo is a Japanese malting barley variety with good agronomic characteristics but is sensitive to waterlogging and salinity (Pang et al., 2004; Zhou et al., 2012).

Seeds were surface sterilized with 10% commercial bleach (NaClO 42 g L^{-1}; Pental Products, Shepparton, Australia), thoroughly rinsed with tap water for at least 30 min and then grown in large containers of size 9×12×6 cm (length×width×height) with basic salt media (BSM) solution (0.5 mM KCl + 0.1 mM $CaCl_2$, pH 5.6) in the dark for 3 day at room temperature (25 ± 1°C). Two treatments were used in this experiment: (1) control (BSM, aerated); and (2) hypoxia (BSM solution made with 0.2% agar and bubbled with N_2 gas). For the treatment with agar, the stagnant solution was prepared by adding agar (Cat. No. LP0011, Oxoid, Hampshire, UK) to the BSM solution at a ratio of 0.2% (w/v) and boiled, then cooled down overnight at a room temperature with magnetic stirring to prevent lump formation. The agar solutions were pre-bubbled with high purity N_2 (Cat. No. 032G, BOC Gases, Hobart, Australia) for at least 1 h before being used in the experiment.

5.1.2 Evaluation of the DH lines for waterlogging and salinity tolerance

All the details related to waterlogging and salt tolerance evaluation experiments are given in our previous publication (Xu et al., 2012). A joint scoring system was used, with scoring index

0 indicating no damage and index 10 given to dead plants. Plants with scores 0-5 showed the various extent of chlorosis and with scores 6 or above had a substantial percentage of necrotic leaves, under waterlogged/salinity conditions.

5.1.3 Determination of hydrogen peroxide and superoxide radical for QTLs

Prior to measurement 3-day old seedlings of barley DH lines were treated with hypoxia solution (0.2% agar) in a container. The container was filled with hypoxia solution while coleoptile being above the surface of the solution. Roots were kept under stagnant conditions for 48 h. The seedlings were then removed from hypoxia solution and ROS species accumulation analysis was done by following the given procedure of staining. Hydrogen peroxide (H_2O_2) accumulation in barley roots of DH lines was detected after the staining with 3,3′-diaminobenzidine (DAB) according to Xu et al. (2010) and Lehotai et al. (2011). In brief, fresh root apices (~0.5 cm) were incubated in 1 mg/mL DAB–HCl solution for 5 h and washed once with 2-*N*-morpholino-ethanesulfonic acid/potassium chloride (Mes/KCl) buffer (10−3 M, pH 6.15). The accumulation of superoxide anion ($O_2^{\cdot-}$) was carried out by using nitro blue tetrazolium (NBT) staining procedure (Lehotai et al. 2011). In this method, root segments (~0.5 cm) were dyed for 2 h with 0.1 mg/mL NBT (in 0.2 M phosphate buffer, pH 7.6) in the dark and then washed once with a phosphate buffer. After staining, the roots were washed with distilled water for 3 to 5 times. All stained roots were observed using a Leica Fluorescence Stereomicroscope (Model MZ16 FA, Leica Microsystems, Heerbrugg, Switzerland) under visible light and photographed with a charge-coupled device (CCD) imaging system attached to the microscope. Then, images were analysed with Image J software (NIH, USA) based on the integrated density. The background intensity of the signal was measured from an empty region with a similar size and subtracted from the whole-cell intensity to obtain relative total cell fluorescence values (Bonales-Alatorre et al., 2013). For each DH line and ROS species, roots segments of at least 6-8 individual seedlings were used for staining after 48 h of treatment; for each of them, between 20 and 30 cell's (technical replicates) intensity values were averaged. For reporting purposes, relative total cell $O_2^{\cdot-}$ and H_2O_2 concentration data shown in Figs 5.1 and 5.2 were divided by 1000.

5.1.4 Genetic map Construction and QTL analysis

Genomic DNA of the DH population was extracted from the leaf tissue of four-week-old seedlings. A total of 28047 DArT and 8928 SNP markers were used for genotyping. After removing markers with greater distortion and missing data, 4788 markers were chosen for map

construction. A new genetic map of the DH population was constructed using the software package JoinMap 4.0 (Van Ooijen, 2006). QTL analysis was conducted using the software package MapQTL 6.0 (Ooijen and Kyazma, 2009). Interval mapping (IM) was firstly used to detect the major QTL. The nearest marker at the QTL from IM was chosen as a cofactor in the multiple QTL models (MQM). The logarithm of the odds (LOD) threshold values applied to declare the presence of a QTL were estimated by performing the genome-wide permutation tests implemented in MapQTL version 6.0 using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold around 3.0. To determine the effects of physiological traits on waterlogging and salinity tolerance, QTL for both waterlogging and salinity tolerance were re-analysed by using various physiological traits as covariates. Maps showing the QTL position and LOD values were generated using MAPCHART (Voorrips, 2002).

5.1.5 Statistical analysis

Statistical analysis was performed by the statistical package IBM SPSS Statistics 21 (IBM, New York, NY, USA). All data in the tables and figures are given as means ±SE. The significant difference between means was evaluated by Duncan's multiple range test. Different lower-case letters represent a significant difference between different cultivars and/or treatments at $P<0.05$.

5.2 Results

5.2.1 ROS ($O_2^{\cdot-}$, H_2O_2) production in barley cultivars under hypoxia stress

Under oxygen-deprived conditions, ROS are produced in plant tissues (Fukao and Bailey-Serres, 2004; Mustroph et al., 2010). To assess the suitability of the staining methodology to quantify this ROS production, six barley cultivars differing in waterlogging tolerance were used in preliminary experiments. Both $O_2^{\cdot-}$ and H_2O_2 showed a genotypic-specific accumulation after 48 h of hypoxia stress (Figs. 5.1, 5.2). The 48 h of hypoxia stress affected the accumulation of $O_2^{\cdot-}$ radical in all cultivars, but to a different extent. The higher accumulation of $O_2^{\cdot-}$ in both elongation and the mature zones was observed in waterlogging sensitive cultivars Gairdner, Franklin, and Naso Nijo (Fig. 5.1A). These visual observations were then quantified by Image J software, revealing statistically significant (at $P < 0.05$) difference between sensitive and tolerant cultivars (Fig. 5.1B, C). The production of $O_2^{\cdot-}$ in both

elongation and mature zones was almost 2- to 2.5-fold higher in waterlogging sensitive cultivars than in tolerant cultivars. For H_2O_2, the intensity of the brown colour was greater in sensitive cultivars after hypoxia, suggesting more H_2O_2 production compared with appropriate controls (Fig. 5.2A). Similarly, sensitive cultivars showed 2.5 to 3-fold higher accumulation of H_2O_2 compared with tolerant cultivars in both elongation and mature zones (Fig. 5.2B, C) when analysed with an Image J software.

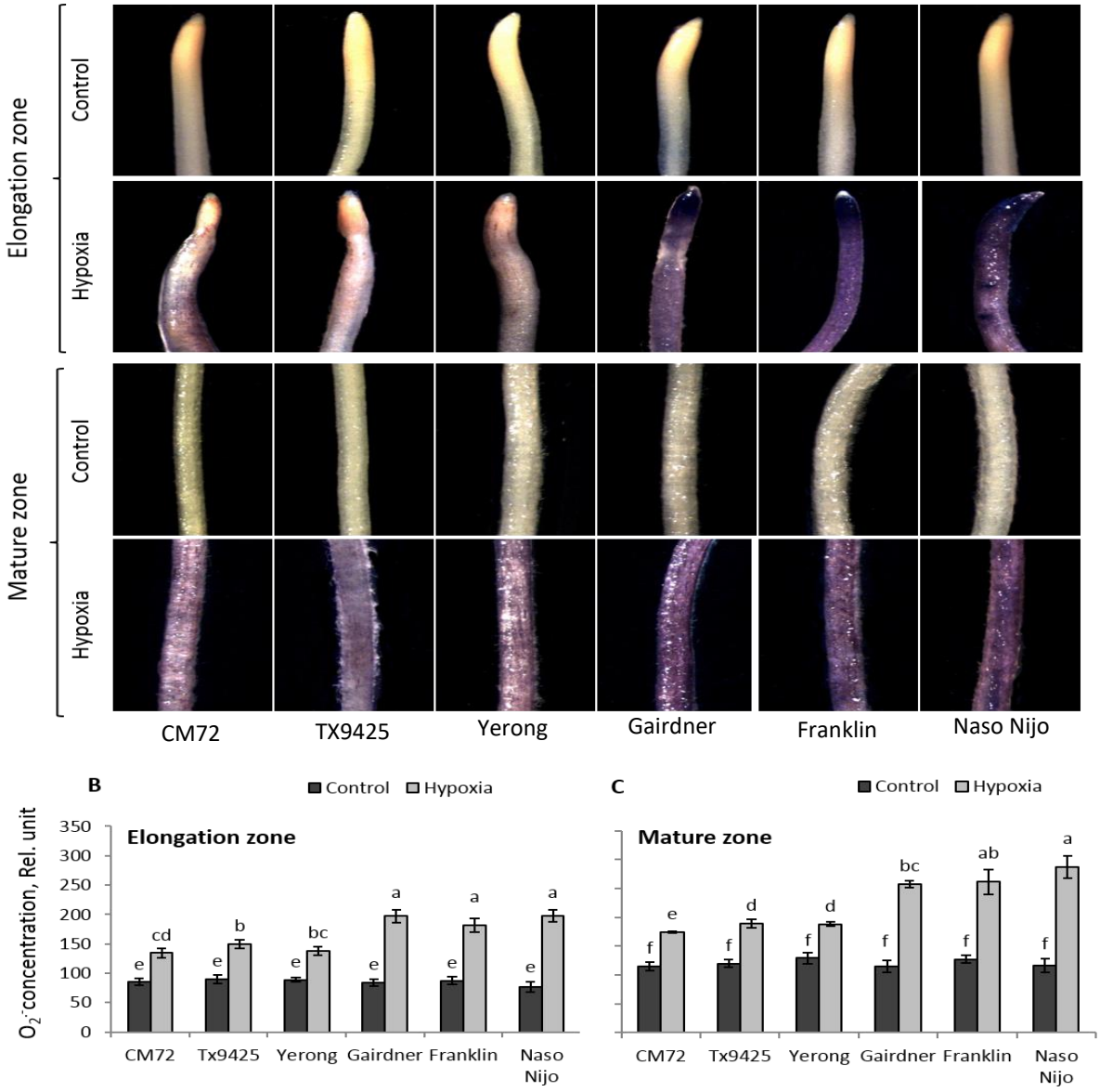

Fig. 5.1 Histochemical detection of superoxide ($O_2^{\cdot-}$) in the elongation and mature zone in the roots of six barley cultivars differing in waterlogging tolerance (A). Relative quantification of the ($O_2^{\cdot-}$) concentration in the elongation (B), and the mature root of barley (C). Relative ($O_2^{\cdot-}$) concentration was calculated by the fluorescence integrated density using Image J software. Data are the mean ± SE [n=150–250; 20–30 cells analysed for at least 6-8 individual seedlings (biological replicates)]. Scale bar = 1 mm.

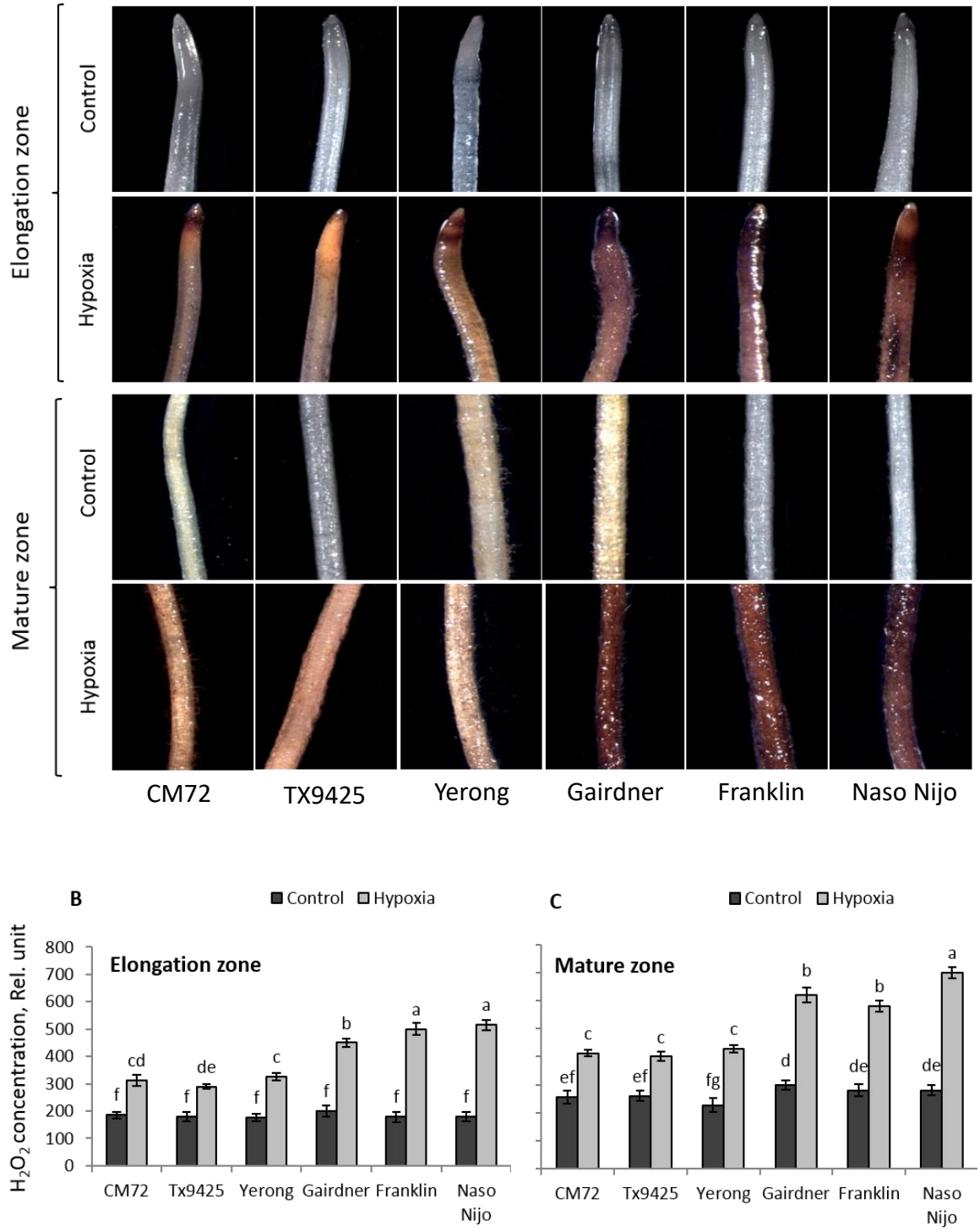

Fig. 5.2 Histochemical detection of hydrogen peroxide (H_2O_2) in the elongation and mature zone in the roots of six barley cultivars differing in waterlogging tolerance (A). Relative quantification of the (H_2O_2) concentration in the elongation (B), and the mature root of barley (C). Relative (H_2O_2) concentration was calculated by the fluorescence integrated density using Image J software. Data are the mean ± SE [n=150–250; 20–30 cells analysed for at least 6-8 individual seedlings (biological replicates)]. Scale bar = 1 mm. Different lowercase letters indicate the significant difference at $P \leq 0.05$ according to Duncan's multiple range tests.

5.2.2 ROS production in DH lines and identification of QTL for ROS tolerance

The DH lines derived from TX9425 and Naso Nijo were used to identify QTL for ROS tolerance under hypoxia stress. Both parent cultivars showed a considerable difference in $O_2^{\cdot-}$ and H_2O_2 production when measured after 48 h of hypoxia in roots (Table 5.1). Under hypoxia stress, the waterlogging sensitive parent Naso Nijo showed a significantly higher accumulation of $O_2^{\cdot-}$ in elongation 197 and mature zone 278 compared with the tolerant parent 149 and 189, respectively in elongation and mature zone (Table 5.1). Similarly, Naso Nijo showed a higher H_2O_2 accumulation in both elongation 515 and mature 691 zones than TX9425 (Table 5.1). Figure 5.3 shows the frequency distribution of ROS tolerance based on the $O_2^{\cdot-}$ and H_2O_2 accumulation. A continuous distribution was found for $O_2^{\cdot-}$ and H_2O_2 accumulation in both elongation and mature zones (Fig. 5.3). A major QTL was identified on chromosome 2H for both $O_2^{\cdot-}$ in mature zone and H_2O_2 in elongation zone (Fig. 5.4). The QTL were designated as (*QSO.TxNn.2H*) for $O_2^{\cdot-}$ and (*QHP.TxNn.2H*) for H_2O_2. The closest marker was 3271162D2 for *QSO.TxNn.2H* and 3999753D2 for *QHP.TxNn.2H*, both at the position of 13.6 cM, explaining 23.7% and 24.1% of the phenotypic variation, respectively (Table 5.2). No significant QTL was identified for $O_2^{\cdot-}$ in elongation zone and H_2O_2 in nature zone under hypoxia, although both showed significant difference among DH lines.

Table 5.1 Effects of hypoxia (0.2% agar) stress on different traits of parents and DH lines. Data are mean values ± S.E.

Cultivars	$O_2^{\cdot-}$ elongation zone	$O_2^{\cdot-}$ mature zone	H_2O_2 elongation zone	H_2O_2 mature zone
TX9424	149.4 ± 7	189.44 ± 6.8	290.7 ± 14.7	400 ± 17.4
Naso Nijo	197.6 ± 10.5	278.62 ± 16.5	515 ± 19.6	691 ± 18.5
DH lines	194.8 ± 14.2	212.12 ± 27.8	416 ± 42.4	576.1 ± 73.3
DH lines range	137.6 - 232.4	135.57 - 287.2	287 - 561.1	358.1 - 777.6

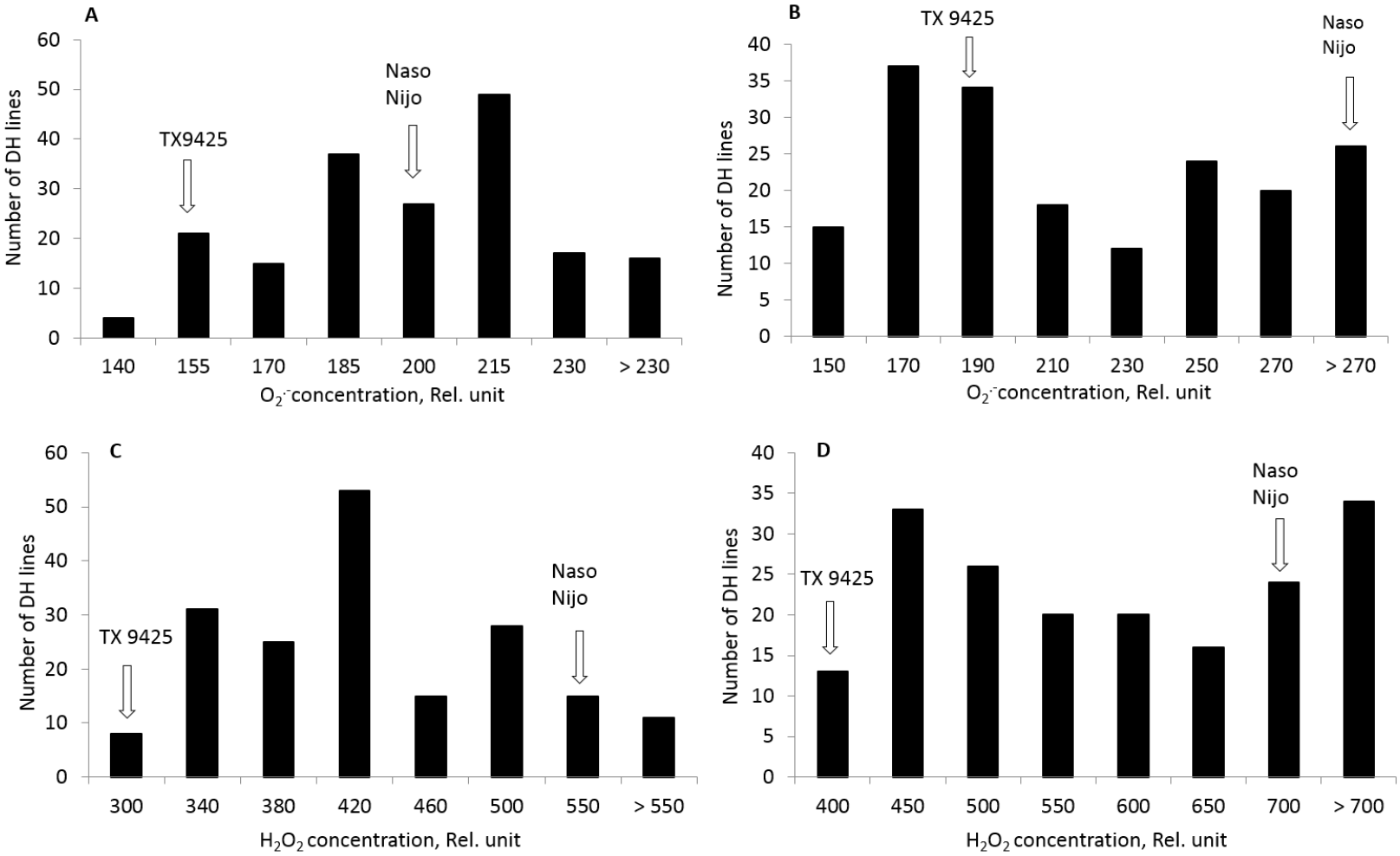

Fig. 5.3 The frequency distribution for superoxide ($O_2^{\cdot-}$) (A, B) and hydrogen peroxide (C, D) under hypoxia (0.2% agar) stress of DH lines derived from a cross of TX9425 and Naso Nijo.

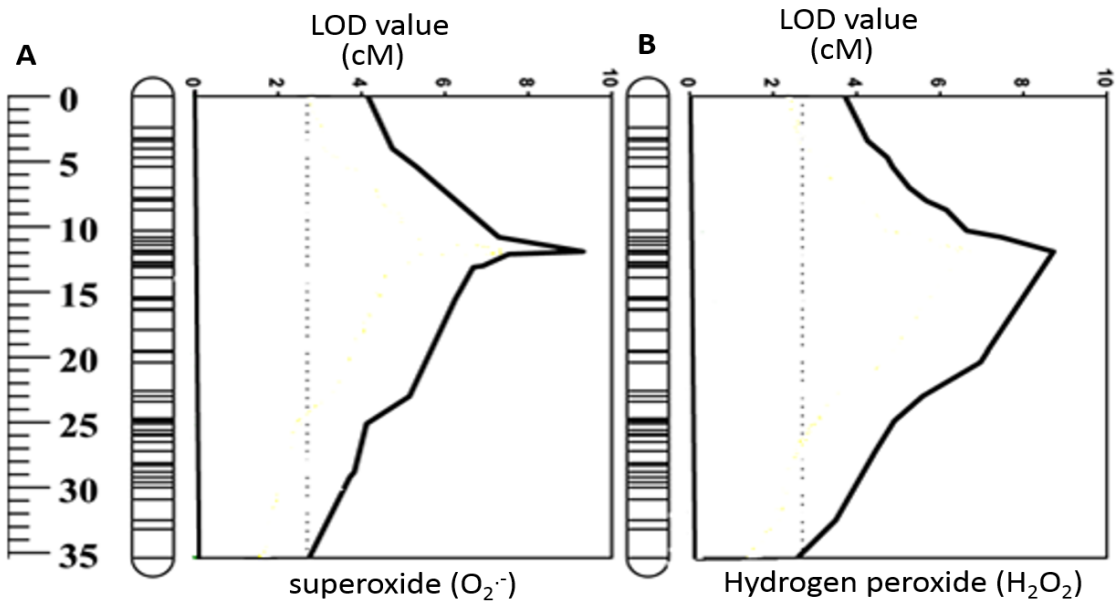

Fig. 4.4 QTL associated with superoxide ($O_2^{\cdot-}$) radical (A), and hydrogen peroxide (H_2O_2) (B). For more clarity, only parts of chromosome regions were shown.

Table 5.2 QTL on 2HS for superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), salt and waterlogging tolerance detected in the DH population of TX9425 × Naso Nijo.

Traits	Linkage group	Nearest marker	Position (cM)	LOD	R^2 (%)	Co-variate
$O_2^{\cdot-}$ Mature zone	2H	3271162D2	13.6	8.7	23.7	
		No QTL identified				waterlogging
		No QTL identified				Salt
H_2O_2 elongation zone	2H	3999753D2	13.6	8.88	24.1	
		No QTL identified				Waterlogging
		No QTL identified				Salt
Waterlogging	2H	3264866S2	9.21	7.61	21	
	2H	3264866S2	9.21	5.6	14.8	O_2
	2H	3264866S2	9.21	5.4	14.3	H_2O_2
Salt	2H	3257177S2	7.79	32.79	63.7	
	2H	3257177S2	7.79	26.7	39.4	O_2
	2H	3257177S2	7.79	26.6	41.3	H_2O_2

5.2.3 Contribution of ROS ($O_2^{\cdot-}$, H_2O_2) to waterlogging and salinity tolerance

The QTLs identified for $O_2^{\cdot-}$ and H_2O_2 in the current study were further used to see their contribution to waterlogging and salinity tolerance by incorporating data published by Xu et al. (2012). The position of identified QTLs in the current study was the same as that for waterlogging and salinity tolerance on chromosome 2H (Xu et al., 2012). Both $O_2^{\cdot-}$ and H_2O_2 showed a significant ($P < 0.05$) correlation with the overall waterlogging tolerance (Fig. 5.5A, B). This is further confirmed by QTL analysis for waterlogging tolerance using $O_2^{\cdot-}$ and H_2O_2 as covariates (Fig. 5.6). As shown in Figure 5.6B, the LOD value of the QTL on 2H for waterlogging tolerance showed a slight reduction when $O_2^{\cdot-}$ and H_2O_2 were used as covariates. The percentage of the phenotypic variation (R^2) determined by the QTL reduced from 21% to 14% when $O_2^{\cdot-}$ was used as a covariate and 21% to 14.3 when H_2O_2 was used as a covariate (Table 5.2). A close and significant correlation ($P < 0.001$) with the salt tolerance was also found for both $O_2^{\cdot-}$ and H_2O_2 (Fig. 5.5C, D). When $O_2^{\cdot-}$ and H_2O_2 were used as covariates, the R^2 of the QTL for salt tolerance reduced from 63 to 39 when $O_2^{\cdot-}$ was used as a covariate and 63 to 41 when H_2O_2 was used as a covariate (Table 5.2).

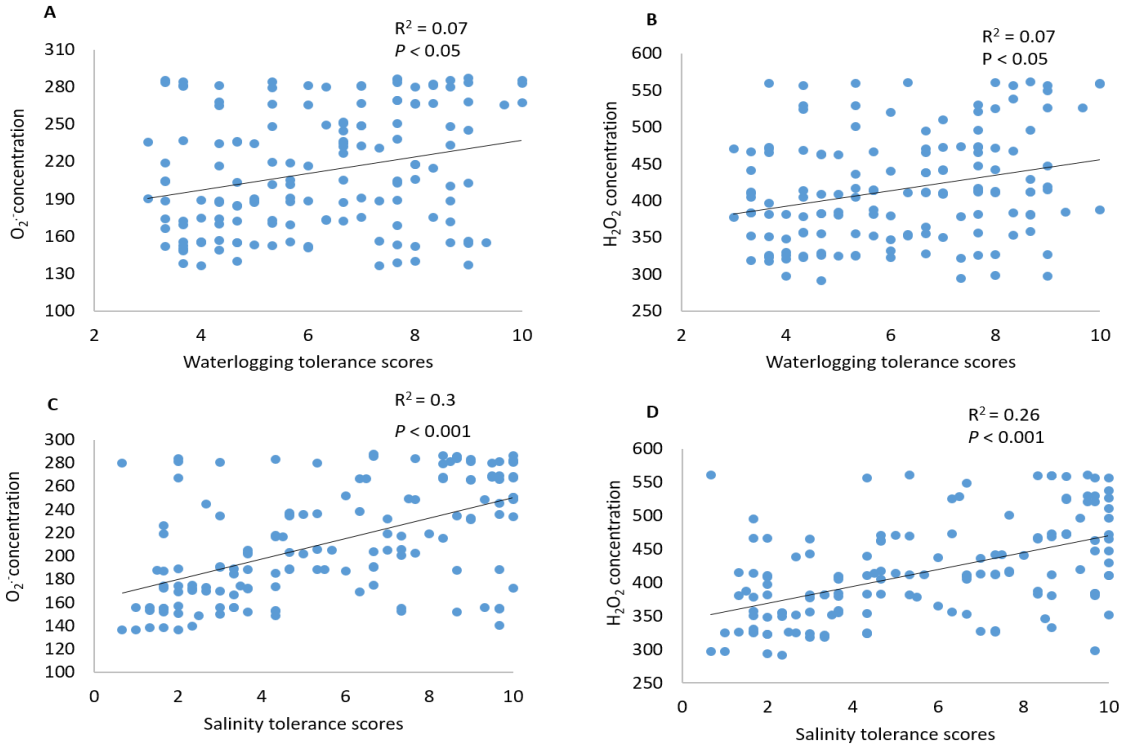

Fig. 5.5 Correlations between superoxide ($O_2^{\cdot-}$) radical concentration in mature zone and waterlogging tolerance scores (A), between hydrogen peroxide (H_2O_2) concentration in elongation zone and waterlogging tolerance scores (B), between superoxide ($O_2^{\cdot-}$) radical concentration in mature zone and salinity tolerance scores (C), and between hydrogen peroxide (H_2O_2) concentration in elongation zone and salinity tolerance scores (D).

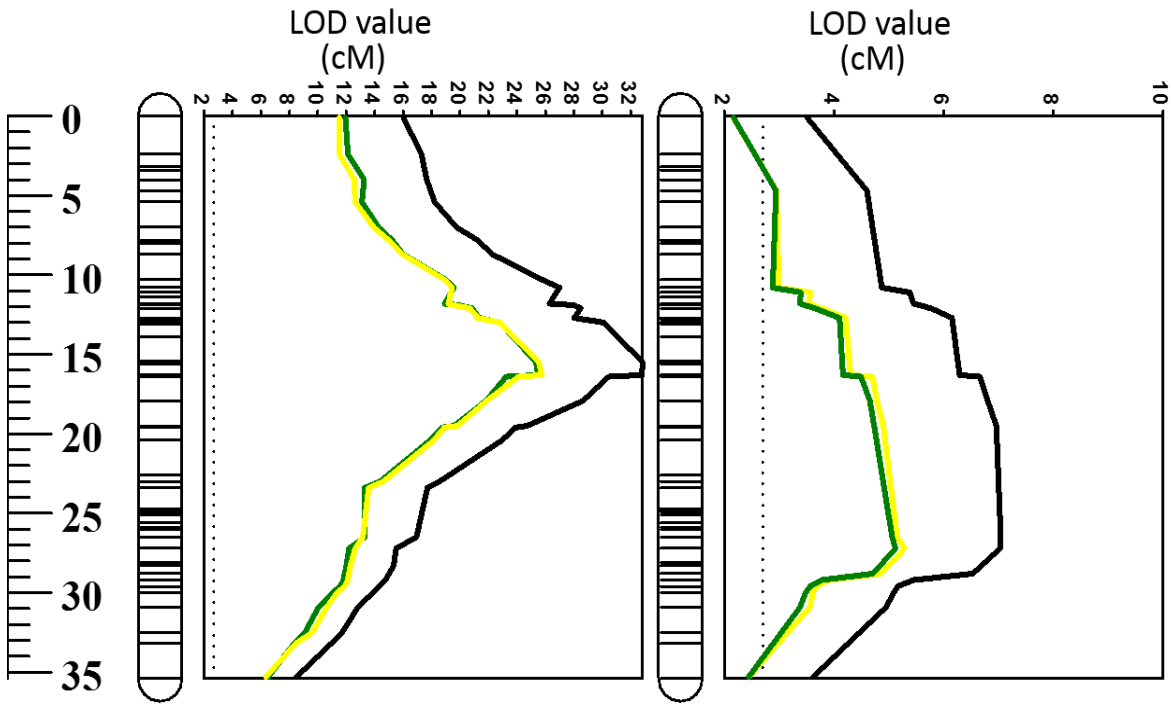

Fig. 4.6 QTL associated with salinity (left) and waterlogging (right) tolerance (LOD values) on 2HS. Black line: LOD value of original QTL; Green line: LOD value of QTL when superoxide ($O_2^{\cdot-}$) in the mature zone was used as a covariate; Yellow line: LOD value of QTL when hydrogen peroxide (H_2O_2) in elongation zone was used as a covariate.

5.2.4 Effects on QTLs for ROS ($O_2^{\cdot-}$, H_2O_2) when using waterlogging and salt tolerance as covariates

These correlation results of ($O_2^{\cdot-}$, H_2O_2) with waterlogging and salinity stress were further confirmed by reverse QTL analysis, i.e. analysis of QTLs for $O_2^{\cdot-}$ and H_2O_2 by using either waterlogging or salt tolerance as covariates (Fig. 5.7; Table 5.2). When such analysis was conducted by using waterlogging tolerance as a covariate, the significance QTL was reduced for $O_2^{\cdot-}$ and H_2O_2 (Fig 5.7; Table 5.2). Similarly, the QTLs for both $O_2^{\cdot-}$ and H_2O_2 became insignificant when salt tolerance scores were used as covariates (Fig. 5.7; Table 5.2).

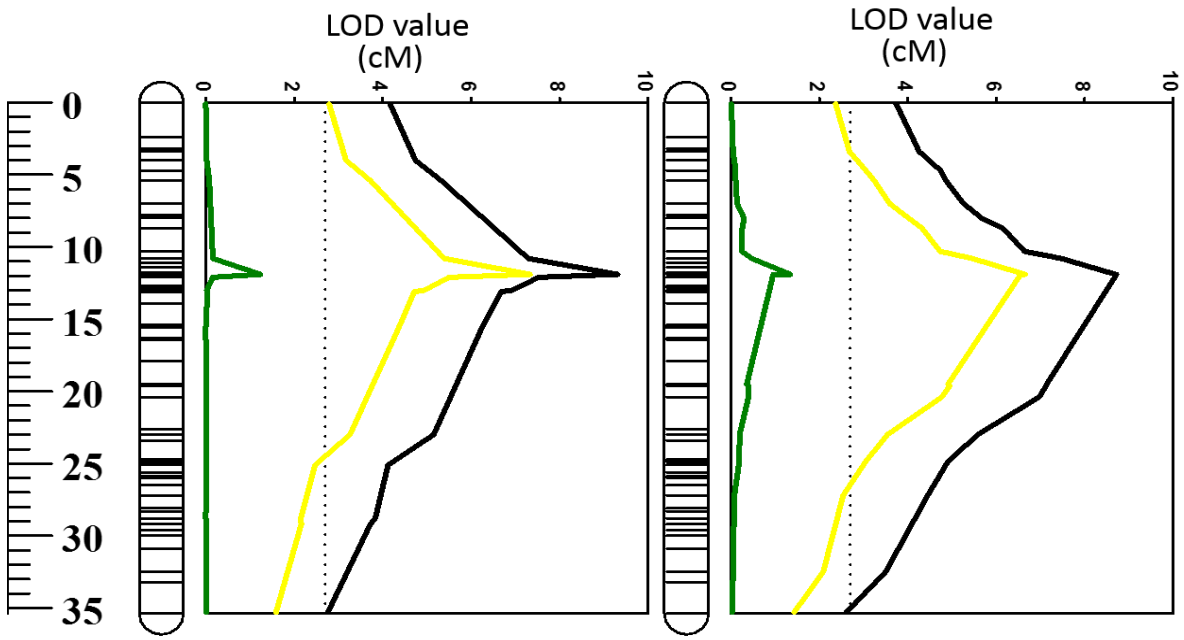

Fig. 4.7 QTL associated with superoxide ($O_2^{\cdot-}$) in the mature zone (left) and hydrogen peroxide (H_2O_2) in elongation zone (right) tolerance (LOD values) on 2HS. Black line: LOD value of original QTL; Green line: LOD value of QTL when salinity tolerance was used as a covariate; Yellow line: LOD value of QTL when waterlogging was used as a covariate.

5.3 Discussion

Waterlogging stress is one of the major abiotic factor limiting agricultural production around the globe. Hence, introducing waterlogging tolerance in field crops is crucial for sustainable food production. Waterlogging tolerance is a complex trait and it can be easily affected by various environmental factors including soil properties, the extent of stress, duration of stress and plant development stage when waterlogging occurs (Mano and Omori, 2007; Zhou, 2011). Due to these confounding factors and low efficiency of adopted direct selection methods, various indirect criteria have been used to select for waterlogging tolerance of plants.

Over the years, many QTL were identified for waterlogging tolerance based on different agronomic, physiological and anatomical traits. In barley, QTL analysis for waterlogging tolerance has been performed based on plant height (Xue et al., 2010), grain yield (Zaidi et al., 2015), plant survival (Zhou et al., 2012), leaf chlorosis (Li et al., 2008; Ma et al., 2015) and plant biomass (Zhang et al., 2013) under waterlogging stress. These QTL were identified on all seven chromosomes, limiting their practical use. Also, most of these studies were based on quantitative traits which can vary between different environments i.e. a QTL detected in one environment could not necessarily be detected in another environment (Beavis, 1998; Van

Kleunen and Fischer, 2005; Mackay et al., 2009). While these traits are convenient for high throughput screening, they may not be directly related to the mechanisms conferring the tolerance. As several QTL are responsible for a trait, fine mapping of these QTL to provide reliable markers to breeders is very difficult.

Recently a more promising approach was adopted, when specific QTL are linked directly with the appropriate mechanisms. The fact that most of the mechanisms are expected to be controlled by just one or two QTL makes it to fine map these mechanisms. A very good example of this success is for barley waterlogging tolerance, the major QTL for waterlogging tolerance on 4H (Li et al., 2008; Zhou, 2011; Zhou et al., 2012) is due to the formation of aerenchyma under stress which is controlled by a single major QTL (Broughton et al., 2015; Zhang et al., 2016; Zhang et al., 2017), and the gene has been fine mapped to a < 2 cM region. The closely linked molecular markers of this gene are available for breeders to use in developing waterlogging tolerance in future breeding programs.

Cellular ROS balance can be disturbed under stress conditions due to either enhanced production of ROS or reduced antioxidants activity in plants (Blokhina et al., 2003; Van Breusegem and Dat, 2006). Under moderate stress conditions, ROS generation primarily acts as a regulatory and adaptive mechanism (Fukao and Bailey-Serres, 2004). For example, ROS signalling plays an essential role in the anatomical adaptations under low oxygen stress by triggering the process of aerenchyma formation (Shabala et al., 2014; Sasidharan and Voesenek, 2015). A recent study also showed the requirement of elevated ROS for PCD during the development of adventitious roots in seedlings of rice (Steffens et al., 2012). However, when stress is severe, excessive generation of ROS damages cellular components and cause their dysfunction. Similarly, H_2O_2 contributes to activating a range of cation-permeable non-selective cation channels (Mori and Schroeder, 2004; Demidchik et al., 2007; Ordonez et al., 2014), thus affecting intracellular K^+ and Ca^{2+} homeostasis (Shabala and Pottosin, 2014), which may initiate PCD. In addition, by interacting with transition metals, H_2O_2 may form hydroxyl radicals, that directly contributes to the activation of GORK channels (Demidchik and Shabala; Rodrigo-Moreno et al., 2013; Demidchik et al., 2014). In the current experiment, hypoxia treated roots showed a significantly higher accumulation of ROS compared with control conditions (Figs. 5.1, 5.2). The accumulation of both $O_2^{\cdot -}$ and H_2O_2 was higher in waterlogging sensitive cultivars than tolerant ones (Figs. 5.1, 5.2; Table 5.1). The DH population also showed a wide range of segregation (Fig. 5.3) the accumulation of $O_2^{\cdot -}$ and H_2O_2 was correlated with

both waterlogging and salinity tolerances. Major major QTL were identified for both $O_2^{\cdot -}$ (*QSO.TxNn.2H*) and H_2O_2 (*QHP.TxNn.2H*) (Fig. 5.4). The QTL is located at the same position on the short arm of chromosome 2H.

Several QTL were reported at this position for different abiotic stress tolerances, which include waterlogging (Zhou, 2011; Xu et al., 2012; Gill et al., 2017), salinity (Xu et al., 2012) and drought (Fan et al., 2015) with some being identified from the same DH population used in this study. Importantly, all these stresses are known to promote the generation and accumulation of excessive ROS in plant tissues (Mueller and Berger, 2009; Møller and Sweetlove, 2010; Suzuki et al., 2011). Therefore, some common mechanisms may contribute to a close relationship between these different stress tolerances. In the current experiment, both $O_2^{\cdot -}$ and H_2O_2 showed significant correlations with waterlogging and salinity tolerance (Fig. 5.5). QTL analysis was also conducted by using other related traits as covariates, which have been proved to be effective in confirming the relationship between different traits (Fan et al., 2015). When $O_2^{\cdot -}$ and H_2O_2 were used as covariates, the QTL for both waterlogging and salt tolerance showed a reduction in both LOD values and R^2 (Fig. 5.6, Table 5.2). The QTL for both $O_2^{\cdot -}$ and H_2O_2 became insignificant after using waterlogging or salt tolerance as covariates (Fig. 5.7; Table 5.2). The fact that QTL were detected for several abiotic stresses at this position of chromosome 2H indicates a specific mechanism for different stress tolerances including waterlogging and salinity tolerance.

Potassium (K^+) is the most abundant inorganic cation in plant cells that plays a significant role in numerous physiological and metabolic processes (Szczerba et al., 2009; Chen and Jiang, 2010). K^+ also plays a role in activating and regulating nearly 70 different metabolic enzymes in plants (Dreyer and Uozumi, 2011; Anschütz et al., 2014). K^+ is considered as a key determinant of the cell fate, by acting as a trigger of the PCD under hostile conditions (Shabala, 2009; Demidchik et al., 2010). At the same time, early studies reported a strong correlation between the ability of plant tissue to retain K^+ and waterlogging stress tolerance (Pang et al., 2006; Zeng et al., 2014). Under hypoxic conditions, K^+ leaked generally through KOR channels. These channels opened due to membrane depolarization and ROS accumulation (Shabala and Pottosin, 2014; Zeng et al., 2014). In our previous study, a major QTL (*QMP.TxNn.2H*) was identified for membrane potential with a 22% phenotypic variation (Gill et al., 2017). Importantly, the position of the QTL was the same as for the QTLs in this

experiment on 2H. The consistent identification of the same region on chromosome 2H in both experiments may point out the presence of a specific common tolerance responsive gene.

In conclusion, to the best of our knowledge, there has been no other report on QTL analysis of waterlogging tolerance based on ROS accumulation. In this study, we have identified a major QTL on chromosome 2H for both $O_2^{\cdot -}$ and H_2O_2 accumulation under waterlogging stress. The position of QTL for ROS was the same as that for waterlogging and salinity tolerance. The fact that only one single QTL was identified makes it easy to fine map the gene responsible for waterlogging/salinity tolerance using this trait as a physiological marker. The molecular markers associated with this QTL may provide valuable evidence for marker-assisted selection (MAS) and for further breeding programs for waterlogging tolerance.

Chapter 6

General discussion and future prospects

6.1 General discussion

Waterlogging stress is among the most important environmental factors threatening cereal production in many parts of the world. Yield losses due to waterlogging stress are estimated at 20-25 % in barley; sometimes these losses can exceed 50 %, depending on the plant developmental stage when the stress occurs. A number of strategies can be adopted to overcome the yield losses due to waterlogging stress; the most cost-effective method, however, is to improve plant waterlogging tolerance through genetic manipulation. The evaluation and introduction of waterlogging tolerance in crops is a complex trait because it is controlled by several complex contributing mechanisms. In order to develop waterlogging tolerance in cereals, it is critical to understand the physiology of flooding tolerance/sensitivity and to identify genes important in mounting a response. Over the years, several morphological, physiological and biochemical approaches were applied to achieve waterlogging tolerance.

Barley is considered to be a waterlogging sensitive cereal (Zhou et al., 2012), although it shows significant variation amongst genotypes (Takeda and Fukuyama, 1986; Zhou, 2010). An advanced genetic approach like quantitative trait loci (QTL) have been intensively used to develop waterlogging tolerant species. Many QTLs for waterlogging tolerance have been reported in previous studies based on different physiological and agronomic traits. For example, QTL mapping was done by targeting aerenchyma formation (Mano and Omori, 2009; Zhang et al., 2015), root porosity (Broughton et al., 2015), grain yield (Zaidi et al., 2015), leaf chlorosis (Li et al., 2008; Zhao et al., 2012; Ma et al., 2015), plant biomass (Zhang et al., 2013) intercellular CO_2 concentration (Liu et al., 2017) and germination rate (Mano and Komatsuda, 2002) as the whole-plant based phenotypic traits. However, none of these findings led to any major progress in creating stress-tolerant cultivars. Several reasons may explain the slow progress in developing waterlogging tolerant cultivars. First, the major shortfall is that in nearly all cases the above phenotyping has been conducted at the whole-plant level, so each of the measured traits was conferred by multiple (and often unrelated) contributing mechanisms. As a result, multiple QTLs have been reported for each of these traits. The second reason is that very often the phenotypic indices used are not directly related to the mechanisms targeted and are, therefore, misleading. Thus, it appears that the real progress in plant breeding can be

achieved only when plant phenotyping will directly target a contributing mechanism. This can be achieved only when such phenotyping is conducted at the cellular level. This thesis contributed to the aforementioned aims, developing physiological and molecular markers for potassium retention, maintenance of negative membrane potential, and accumulation of reactive oxygen species (ROS) – critical traits for the waterlogging tolerance which have never been targeted in previous breeding programmes.

The first major problem under waterlogging conditions is the absence of oxygen. This oxygen depletion under waterlogged conditions results in a compromised operation of H^+ -ATPase with strong implications for membrane potential maintenance, cytosolic pH homeostasis, ROS tolerance, plant ionic homeostasis (e.g. potassium retention) and transport of all nutrients across membranes. In the first part of this study, a glasshouse experiment was conducted with six barley cultivars under waterlogging stress. After four weeks of waterlogging stress, the most severe effects of waterlogging on plant growth were observed in sensitive cultivars. These six cultivars were used in further MIFE experiments. The results of this study showed a tissue- and genotype-specific effects of hypoxia on the K^+ retention in roots of barley. Hypoxia-induced K^+ fluxes in the mature zone were considerably different from the elongation zone and a time-dependent progressive decline in K^+ uptake is observed in sensitive genotypes, as hypoxia stress progressed. Also, the significant role of voltage-gated K^+-permeable channels (GORK) channel in K^+ release was investigated by measuring hypoxia-induced changes in root membrane potential and correlating it with the extent of K^+ efflux from the root. Our results showed that the genotypic difference in waterlogging stress tolerance in barley is also contributed by the differential ability to regulate voltage-gated K^+-permeable channels in the mature root epidermis. In addition, a strong positive correlation between the ability of mature zone cells to retain K^+ and the overall waterlogging stress tolerance makes it possible to recommend using this method as a physiological marker for breeding plants for waterlogging stress tolerance. Our results suggested that the K^+ flux measured in mature root zone in both control and treated plants was uniform and sensitive enough to discriminate between tolerant and sensitive cultivars. Given that measurements are conducted in a steady state, each of them requires only 1.5-2 min allowing ~30 to 40 specimens be measured in one hour.

Plant abiotic stress tolerance is conferred by many interrelated mechanisms. Amongst these, the cell's ability to maintain membrane potential is considered to be amongst the most

crucial traits, a positive relationship between the ability of plants to maintain highly negative membrane potential and its tolerance to waterlogging stress. However, no attempts have been made to identify quantitative trait loci (QTL) conferring this trait. The suggested protocol in our previous experiment was applied to screen a large number of double haploid (DH) populations for developing molecular markers and mapping QTLs for waterlogging tolerance. For screening DH lines, the microelectrode MIFE technique was used to measure the plasma membrane potential of epidermal root cells of 150 double haploid (DH) lines of barley from a cross between a Chinese landrace TX9425 and Japanese malting cultivar Naso Nijo under hypoxic conditions. A major QTL for the membrane potential in the epidermal root cells in hypoxia-exposed plants was identified. This QTL was located on 2H, which was designated as *QMP.TxNn.2H* and the position of this QTL was close to the 8613801D2 marker at 8.85 cM and explained 22% of the phenotypic variation. This QTL was located at a similar position to the QTL for waterlogging and salinity tolerance reported in previous studies. Further analysis confirmed that membrane potential showed a significant contribution to both waterlogging and salinity tolerance. The fact that the QTL for membrane potential was controlled by a single major QTL illustrates the power of the single-cell phenotyping approach and opens prospects for fine mapping this QTL and thus being more effective in marker-assisted selection.

A reduced concentration of oxygen in waterlogged soils leads to oxygen deficiency in plant tissues, resulting in an excessive accumulation of ROS in plants. To identify QTL for ROS tolerance in barley, 187 double haploid (DH) lines from a cross between TX9425 and Naso Nijo were screened for superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) accumulated under hypoxia stress. In our experiment, we showed that quantifying ROS contents after 48 h hypoxia could be a fast and reliable approach for the selection of waterlogging tolerant barley genotypes. Two major QTL on chromosome 2 were identified each for $O2^{\cdot-}$ (*QSO.TxNn.2H*) and H_2O_2 (*QHP.TxNn.2H*) contents. The QTL were designated as (*QSO.TxNn.2H*) for $O_2^{\cdot-}$ and (*QHP.TxNn.2H*) for H_2O_2. These QTL were located at the same position as the QTL for the overall waterlogging and salt tolerance reported in previous studies, explaining 23% and 24% of the phenotypic variation, for $O_2^{\cdot-}$ and H_2O_2 contents, respectively. The analysis also showed a causal association between ROS production and both waterlogging and salt stress tolerance. The markers associated with this QTL could potentially be used in future breeding programs to improve waterlogging and salinity tolerance.

6.2 Future prospects

This PhD project was an integral part of an Australian Research Council Linkage grant to develop physiological and molecular markers for barley breeding under waterlogging stress. Among targeted barley cultivars, TX9425 was found to be the most waterlogging tolerant, with the least reduction in plant growth, better K^+ retention and ability to maintain a higher membrane potential. On the other hand, Naso Nijo was found to be the most susceptible to waterlogging stress. These two cultivars were used as the parent lines to produce DH population in our research group. In this study, the lines of this DH population were used to develop markers for barley breeding. Significant developments have been made to develop waterlogging tolerance in barley during this study. However, there are several questions still need to be addressed in future studies including:

1. The data reported in the present study implicates cytosolic K^+ retention as a key determinant of plant adaptive ability to hypoxia stress. The roots of barley showed a tissue-specific transport and regulation of K^+ under oxygen-limited conditions. This may happen due to a difference between concentration of supply and requirement of root tissues for oxygen. Here we need a study to measure the actual oxygen concentration at a specific cell and root zone. This may help to narrow down the responsible genes for waterlogging tolerance.
2. Oxygen-deficient conditions lead towards energy crises by limiting O_2 availability for ATP production, which results in a limited supply of energy to fuel H^+-ATPase pumps that enable H^+ extrusion. As a result, the channel-mediated uptake of many essential cations is reduced. At the same time, depolarization-activated channels increase the leakage of some cations from plant tissues. It is proven in literature and also shown in our study that the role of H^+-PPase becomes critical when H^+-ATPase pumps are not fully functional. Thus, the real progress can be made by enhancing the plant's ability to maintain sufficient supply of oxygen for the operation of H^+-ATPase or its ability to switch to H^+-PPase for active H^+ extrusion, to avoid acidosis.
3. In this study, a major QTL for membrane potential and two major QTL based on ROS accumulation were identified for waterlogging tolerance, but, due to limited resolution of QTL mapping, the associated markers with these novel QTL may not be used to tag genes for waterlogging tolerance. The best solution of this problem

may be to produce near-isogenic lines (NILs) to identify the key regulator genes. NIL-derived populations make it possible to fine map a QTL.The other possibility is to use knockout lines by using rice, in understanding the importance of the candidate genes within these QTL, especially that on chromosome 2H. The fact that the positions of these QTL were the same on chromosome 2H makes it easy to fine map the gene responsible for waterlogging tolerance. The molecular markers associated with this QTL may provide valuable evidence for marker-assisted selection (MAS) and facilitate work on map-base gene cloning in future breeding programs.

4. The other possible opportunity is to conduct a detailed bioinformatic analysis to find any homology or collinearity between the QTL (*Sub1*), a major QTL underlying submergence in rice and the QTL *QMP.TxNn.2H* for maintenance of negative membrane potential, and accumulation of ROS identified in this study.

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